INVESTIGATION OF ARCHETYPE HUMAN POLYOMAVIRUS JC CELLULAR TROPISM AND GENOMIC ALTERATIONS IN JC VIRUS PATHOGENESIS

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ABSTRACT

The human polyomavirus JC (JCPyV) is the causative agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). While the archetypal form of the virus is ubiquitous in the healthy human population, it is the rearranged form that is responsible for PML. The archetype form of JCPyV has a conserved noncoding control region (NCCR) that is defined by six designated blocks, A-F. However, the rearranged form has deletions and/or duplications in its NCCR. Although it has been established that the rearranged form of JCPyV is pathogenic, the events leading to the reactivation and/or rearrangement in its NCCR have yet to be determined. Thus, the lack of *in vitro* and *in vivo* archetype JCPyV replication models have hindered the understanding of mechanisms underlying the development of PML pathogenesis. In this report, we demonstrate *in vitro* infection and efficient replication of archetype JCPyV in renal proximal tubule epithelial (RPTE) and human brain microvascular endothelial (HBMVE) cells, limited or no replication in human brain cortical astrocytes (HBCA) and primary human fetal glial (PHFG) cells, and *in vitro* rearrangement of archetype JCPyV at day 645 in COS-7 cells. In addition, we demonstrate that archetype JCPyV (CY) and rearranged JCPyV (Mad1) can replicate in HBMVE cells, while limited replication was observed when HBMVE cells were transfected with the hybrid JCPyV (CYrM1c). Lastly, we demonstrate *in vivo* infection of JCPyV in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice. To our knowledge, this is the first study demonstrating the ability for urine-derived archetype JCPyV to rearrange *in vitro*, to be infectious in naïve primary cells, and to demonstrate JCPyV infection in humanized NSG mice. This study will therefore give insight on cellular conditions involved in urine-derived archetype JCPyV infection, reactivation, and rearrangement, which will impact the development of much-needed therapeutics for PML.

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ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
APyV	avian polyomavirus
BatPyV	bat polyomavirus
BPyV	bovine polyomavirus
BKPyV	Brennan Krohn polyomavirus
CaPyV	canary polyomavirus
cDNA	complementary deoxyribonucleic acid
CPyV	crow polyomavirus
JCPyV	John Cunningham polyomavirus
FPyV	finch polyomavirus
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GHPyV	goose hemorrhagic polyomavirus
HaPyV	hamster polyomavirus
HBCA	human brain cortical astrocytes
HBMVE	human brain microvascular endothelial cells
HIV	human immunodeficiency virus
HPyV6	human polyomavirus 6
HPyV7	human polyomavirus 7
IFA	immunofluorescence assay
IP	immunoprecipitation
IRIS	immune reconstitution inflammatory syndrome
KIPyV	Karolinska Institute polyomavirus
LPyV	B-lymphotropic polyomavirus
mAbs	monoclonal antibodies

MCPyV	Merkel cell polyomavirus
MPtV	murine pneumotropic virus
MPyV	murine polyomavirus
MS	multiple sclerosis
MWPyV	Malawi polyomavirus
NCCR	non-coding control region
ori	origin of replication
OraPyVI	Bornean orangutan polyomavirus
OraPyV2	Sumatran orangutan polyomavirus
PCR	polymerase chain reaction
PHFG	primary human fetal glial cells
PML	progressive multifocal leukoencephalopathy
PyV	polyomavirus
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
SA12	baboon polyomavirus
SLPyV	California sea lion polyomavirus
SqPyV	squirrel monkey polyomavirus
SV40	simian vacuolating virus 40
TCR	transcriptional control region
TSPyV	trichodysplasia spinulosa-associated polyomavirus
VP1	viral capsid protein 1
WUPyV	Washington University polyomavirus
WB	western blot

CHAPTER 1

BACKGROUND

JC VIRUS AND HUMAN DISEASE

JCPyV

JC virus (JCPyV), a neurotropic human polyomavirus belonging to the genus *Orthopolyomavirus* in the family *Polyomaviridae*, was first isolated in 1971 from the brain of John Cunningham, a patient suffering from progressive multifocal leukoencephalopathy (PML), for whom the virus is named (183). Polyomaviruses have been found in humans, monkeys, rodents and birds (48, 105). According to the International Committee on Taxonomy of Viruses, the family *Polyomaviridae* consists of three genera *Orthopolyomavirus* with the species simian vacuolating virus 40 (SV40), Brennan Krohn polyomavirus (BKPyV), and JCPyV; *Wukipolyomavirus* with Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV); and *Avipolyomavirus* with respective PyVs infecting birds (Fig.1) (111) . In addition to JCPyV, the polyomaviruses that have the ability to infect humans include BKPyV, KIPyV, WUPyV and Merkel cell polyomavirus (MCPyV), Trichodysplasia spinulosa-associated polyomavirus (TSPyV), human polyomavirus 6, 7, and 9 (HPyV6, HPyV7, and HPyV9), and Malawi polyomavirus (MWPyV) (117, 231). The prototype nonhuman primate polyomavirus, simian vacuolating virus 40 (SV40), is also known to rarely infect humans.

Like other tumor viruses in the family *Polyomaviridae*, JCPyV is non-enveloped with an icosahedral capsid containing a small, circular, double-stranded DNA genome. JCPyV virions measure approximately 40-45 nm in diameter and it's circular double-stranded DNA genome is 5.1 kb (147). The single negatively super coiled double-stranded DNA is associated with host cell histones to form mini-chromosomes.

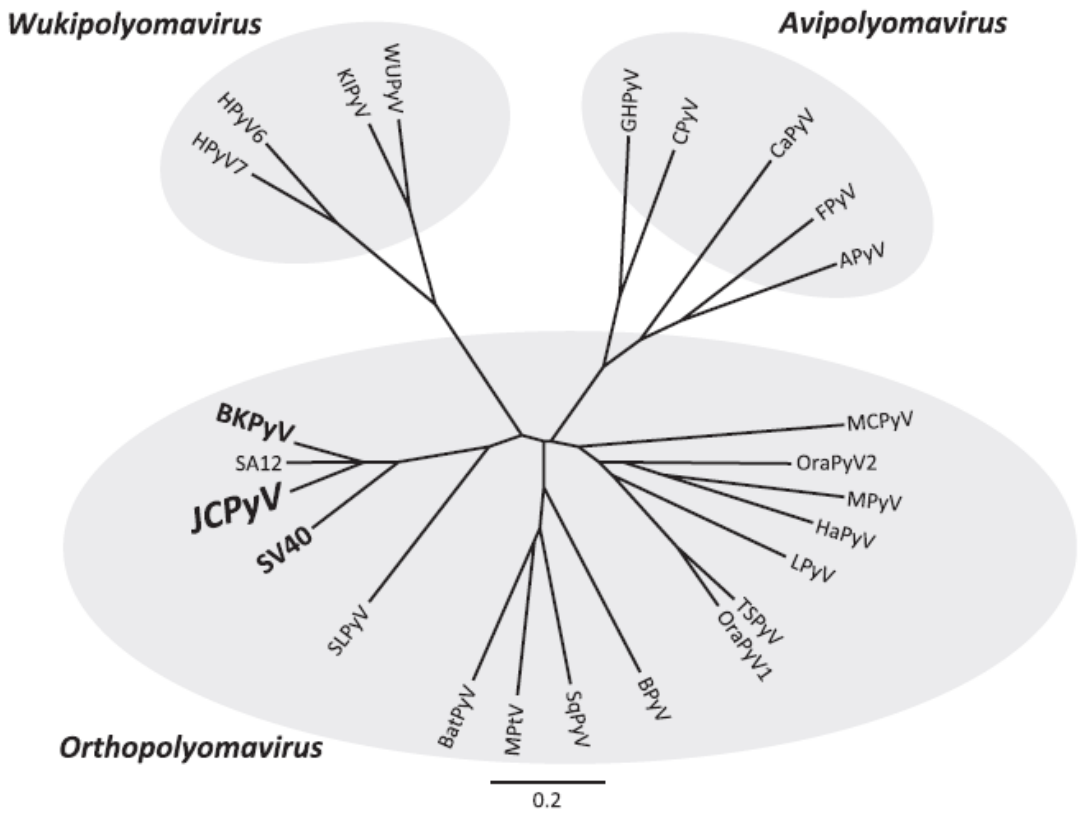

Figure 1. Phylogenetic tree of Polyomaviridae: The phylogenic relationships among the *Polyomaviridae* family was constructed based on whole genomic nucleotide sequences (99). The mammalian polyomaviruses include the genera *Orthopolyomavirus* and *Wukipolyomavirus*, while polyomaviruses infecting birds are grouped under the *Avipolyomavirus* genus. JC polyomavirus (JCPyV), BK polyomavirus (BKPyV), Simian virus 40 (SV40), Baboon polyomavirus I (SA12), California sea lion polyomavirus (SLPyV), Bat polyomavirus (BatPyV), Murine pneumotropic virus (MPtV), Squirrel Monkey polyomavirus (SqPyV), Bovine polyomavirus (BPyV), Bornean orangutan polyomavirus (OraPyVI), Tricodysplasia spinulosa-associated polyomavirus (TSPyV), B-lymphotropic polyomavirus (LPyV), Hamster polyomavirus (HaPyV), Murine polyomavirus (MPyV), Sumatran orangutan polyomavirus (OraPyV2), Merkel cell polyomavirus (MCPyV), Human polyomavirus 6 (HPyV6), Human polyomavirus 7 (HPyV7), Karolinska Institute polyomavirus (KIPyV), Washington University polyomavirus (WUPyV),

Goose hemorrhagic polyomavirus (GHPyV), Crow polyomavirus (CPyV), Canary polyomavirus (CaPyV), Finch polyomavirus (FPyV), Avian polyomavirus (APyV)

JCPyV's circular genome is functionally divided into three regions: the early coding region, the late coding region and the non-coding control region (NCCR) or regulatory region (Fig.2) (145). Transcription of the early and late coding regions begin at the NCCR (0.4 kb), where early transcription proceeds in a counterclockwise direction and late transcription proceeds clockwise on the opposite strand of the DNA (117). The NCCR encompasses the origin of replication (ori), viral promoter-enhancing sequences, and the transcriptional control region (TCR), which act as binding sites for cell transcription factors (43).

The early region (2.4 kb) encodes the transforming proteins, large T and small t antigens, which are involved in gene regulation, viral replication and important in promoting transformation of cells in culture and oncogenesis in vivo (117, 230); along with the recently described T' proteins generated by alternative splicing of the early mRNA.

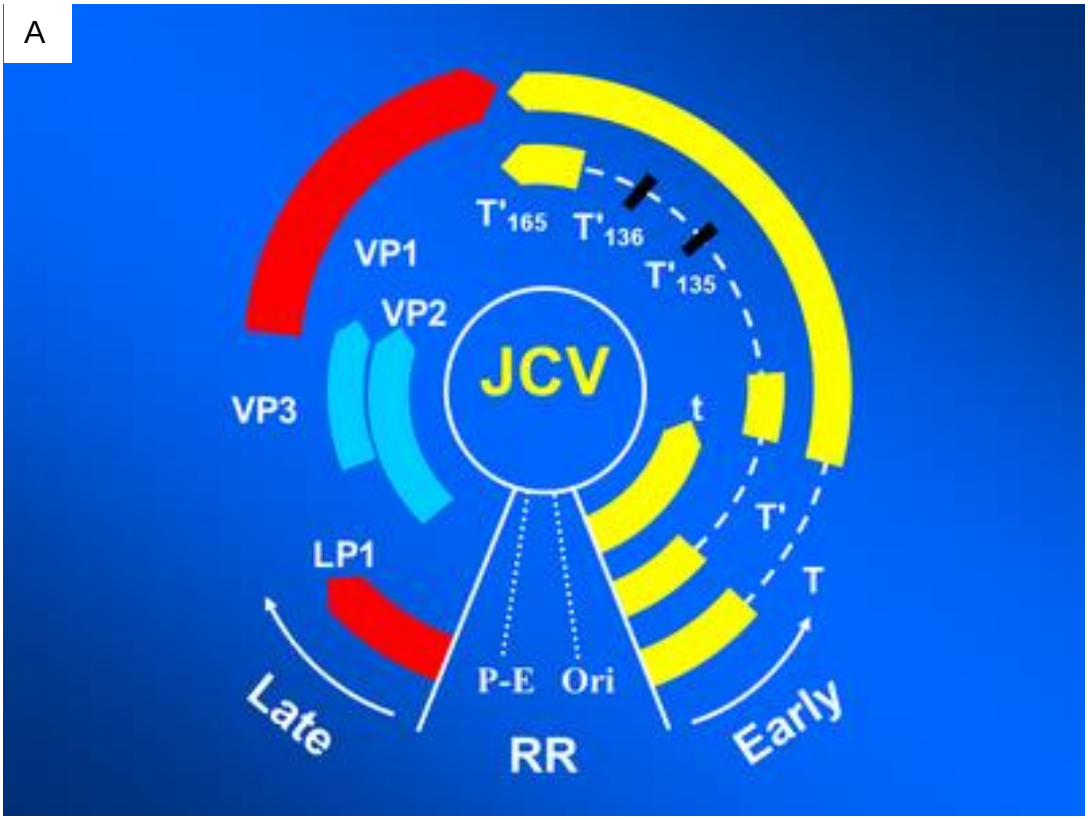

Figure 2. JCPyV genome: **(**A) The JC virus circular double stranded DNA genome is 5.13 Kb and consists of three regions: the early coding region, the late coding region, and the non-coding control region (NCCR) or regulatory region (78, 84). (B) The archetype NCCR is usually found in the kidney and urine of both healthy and immunosuppressed individuals. Rearranged strains are characterized by having deletions, duplications, and/or tandem repeats in their NCCRs, and are most commonly found in the brain or cerebrospinal fluid of PML patients.

The late region (2.3 kb) encodes the major viral capsid protein VP1 which mediates cell attachment, the minor capsid proteins VP2 and VP3, and the accessory agnoprotein (43). The early and late coding regions are highly conserved and have not been convincingly associated with disease pathogenesis (230). They do however, make up about 90% of the viral genome and confer the genotype that is associated with the various subtypes that can be found in different geographical areas (230).

Based upon the structure of the NCCR, two types of JCPyV have been identified in human tissues. The sequence of the regulatory region is known as the “archetype”, because it is thought that all other forms of JCPyV have evolved from it (260). Archetype JCPyV is found in the kidneys and urine of healthy individuals as well as those affected with PML (139, 260) and it is this type that is thought to circulate in the human population. Little sequence variation exists in the genomes of independent isolates of archetype JCPyV (259, 260). It has been suggested that the rearranged form is generated by sequence rearrangements within the archetype NCCR during viral replication, yielding a new, potentially more active form of the virus (50). The regulatory region of JCPyV most often isolated from CSF and brain of patients with PML has rearrangements, including duplications, tandem repeats, insertions and deletions (Fig.3). The rearranged form can also be detected in brain, tonsil and lymphocytes in people with and without PML (81, 213). The differences in cellular tropism of both archetype and rearranged JCPyV in PML and non-PML patients are summarized in Table 1.

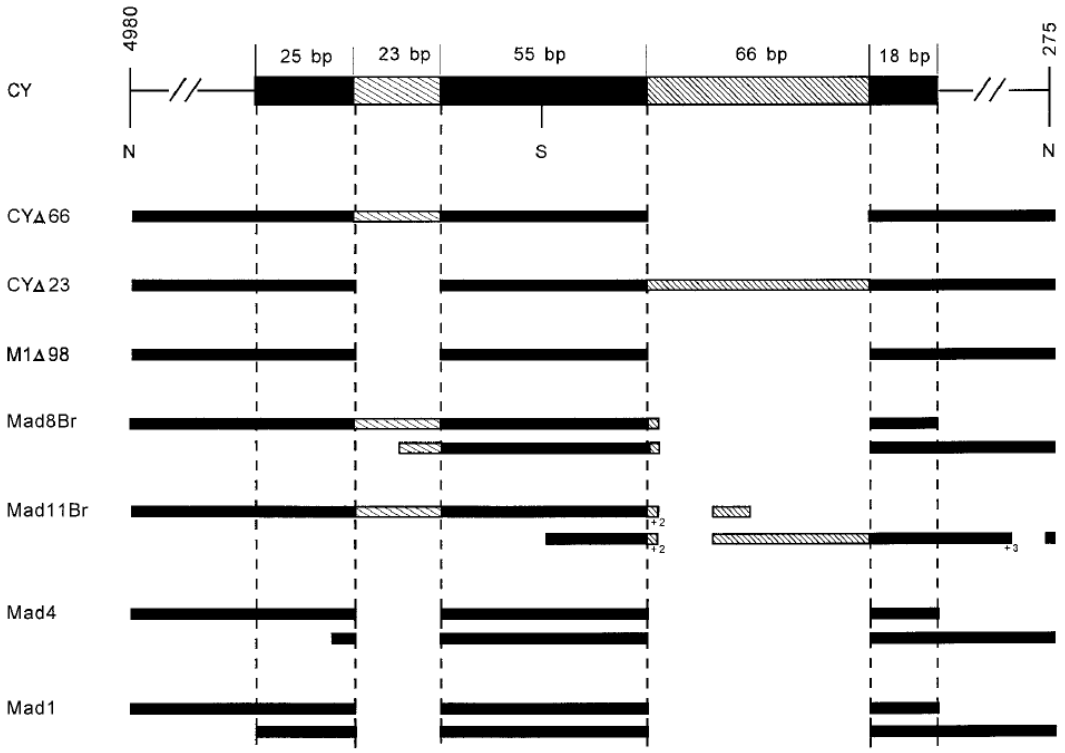

Figure 3. Structural arrangements of various JCPyV NCCRs: Vertical numbers above the diagram indicate the nucleotide position according to the numbering system described in Frisque *et al*. 1984 (79). Horizontal numbers indicate the number of nucleotides defined in each block of the NCCR archetype region, with the TATA box contained in the 25-bp region. The CY strain of JCPyV was first isolated from the urine of a healthy individual and described by Yogo *et al.*, 1990 (260). Mad1, Mad4, Mad11Br, and Mad8Br are naturally occurring strains isolated from PML brain tissue described by Padgett *et al.* 1971 (183) and Grinnell *et al.* 1983 (90). CYΔ23, CYΔ66, and MΔ198 are laboratory constructs (49). Sequences that are identical to the CY strain are represented by solid horizontal lines, deleted sequences by gaps between lines, and duplications by parallel lines. Repeated sequences are read from the beginning of the sequence from the left and continues to the right before continuing at the left of the second line.

Table 1. JCPyV tropism in PML and non-PML patients

Non-PML patients	References	PML patients	References
Archetype JCPyV in urine	Kitamura *et al.* (1990) (121), Markowitz *et al.* (1993) (155), Omodeo-Zorini *et al.* (2003) (180)	Archetype JCPyV in urine	Dorries *et al.* (1983) (55), White *et al.* (1992) (250)
		Archetype and rearranged JCPyV in bone marrow	Marzocchetti *et al.* (2008) (159)
		Rearranged JCPyV in plasma	Fedele *et al.* (2003) (66)
Archetype JCPyV in B lymphocytes		Archetype JCPyV in B lymphocytes	Houff *et al.* (1988) (102)
Rearranged JCPyV in lymphocytes*	Tornatore *et al.* (1992) (234)	Rearranged JCPyV in lymphocytes	Tornatore *et al.* (1992) (234)
Archetype JCPyV in kidneys	Chesters *et al.* (1983) (39), Kitamura *et al.* (1997) (123)		
Archetype JCPyV in gastrointestinal tract	Laghi *et al.* (1999) (131), Selgrad *et al.* (2009) (211), Ricciardiello *et al.* (2000) (193)		
Archetype JCPyV in tonsillar tissue	Goudsmit *et al.* (1981) (88), Kato *et al.* (2004) (112)		
Archetype JCPyV in brain	White *et al.* (1992) (250), Perez-Liz *et al.* (2008) (186)	Archetype and rearranged JCPyV in brain	White *et al.* (1992) (250), Tan *et al.* (2010) (229)
Archetype JCPyV in CNS and CSF*	Vago *et al.* (1996) (237)	Rearranged JCPyV in the CNS and CSF	Vago *et al.* (1996) (237), Fedele *et al.*l (2006) (67)

*HIV-1 positive patients without PML

JCPyV transmission and epidemiology

Although JCPyV is widespread throughout the human population, the mechanism of human-to-human transmission of JCPyV is poorly understood. Seroepidemiological data indicate that JCPyV infection occurs during childhood and is typically subclinical (182). Furthermore, about 80-85% of the adult population have antibodies against JCPyV, which implies previous exposure and a possible latent infection (83, 150). It is known that JCPyV is excreted and found in sewage which suggests oral ingestion and inhalation of contaminated material as a possible entry of JCPyV into the human population (19, 169). Recent environmental studies demonstrated detection of human polyomaviruses (HPyV) in almost all types of environmental water, including wastewater (25, 120), costal seawater (171), storm water (218), river water (26, 93, 97), and drinking water sources (2).

A possible means by which JCPyV enters and spreads in the body is via the infection of tonsil cells. It is presumed that via infected tonsil cells, JCPyV subsequently spreads elsewhere by replication in lymphoid cells (169). Because asymptomatic shedding of JCPyV in the urine can be seen in both healthy individuals and immunosuppressed patients (4) the kidney is thought to be the major organ of JCPyV persistence during latency (39). After initial infection, the virus disseminates and establishes a persistent infection in the kidney throughout life. It is thought that upon reactivation, JCPyV enters the brain via a Trojan horse mechanism via B lymphocytes.

JCPyV life cycle

JCPyV infects cells by first binding to a receptor on the outer membranes of susceptible cells. JCPyV possess intrinsic hemagglutination activity which allows it to engage alpha 2-3- and/or alpha 2-6-linked sialic acid residues, suggesting binding to oligosaccharide as an important step in JCPyV infection (136). It has also been shown that JCPyV can interact with the serotonin receptor 2A (5HT2AR) (62), which leads to virus internalization into glial cells. Virus is taken up by clatherin-dependent endocytosis (10) followed by its transportation to the nucleus where the removal of the viral capsid proteins occurs. Early transcription results in a primary transcript that is alternatively spliced into two mRNAs which code for the large T-antigen (TAg), a nuclear phosphoprotein that is essential for viral DNA replication, and the small t-antigen (117). TAg complexes with host DNA polymerase α and the replication protein-A at the origin of DNA replication to promote DNA synthesis (18). Once TAg initiates DNA replication and stimulates transcription from the late promoter the late phase of the viral lifecycle begins. JCPyV relies on host cell enzymes and cofactors for DNA replication. Since expression of these proteins are confined to the S-phase of the cell cycle, TAg stimulates the cell cycle by modulating cellular signaling pathways via binding key cellular control proteins including p53, pRb, and IRS-1, for example (253). Hsp70 interacts with VP2, VP3, and TAg and accumulates in the nucleus of infected cells. Association of VP2, VP3, and TAg though their DNA binding domains results in enhancing TAg binding to the origin of replication subsequently inducing JCPyV viral DNA replication (208). Ultimately, the capsid proteins, VP1, VP2 and VP3, are expressed from the late region and assemble with the replicated viral DNA to form intranuclear virions, which are released upon cell lysis (60, 117) (Fig.4).

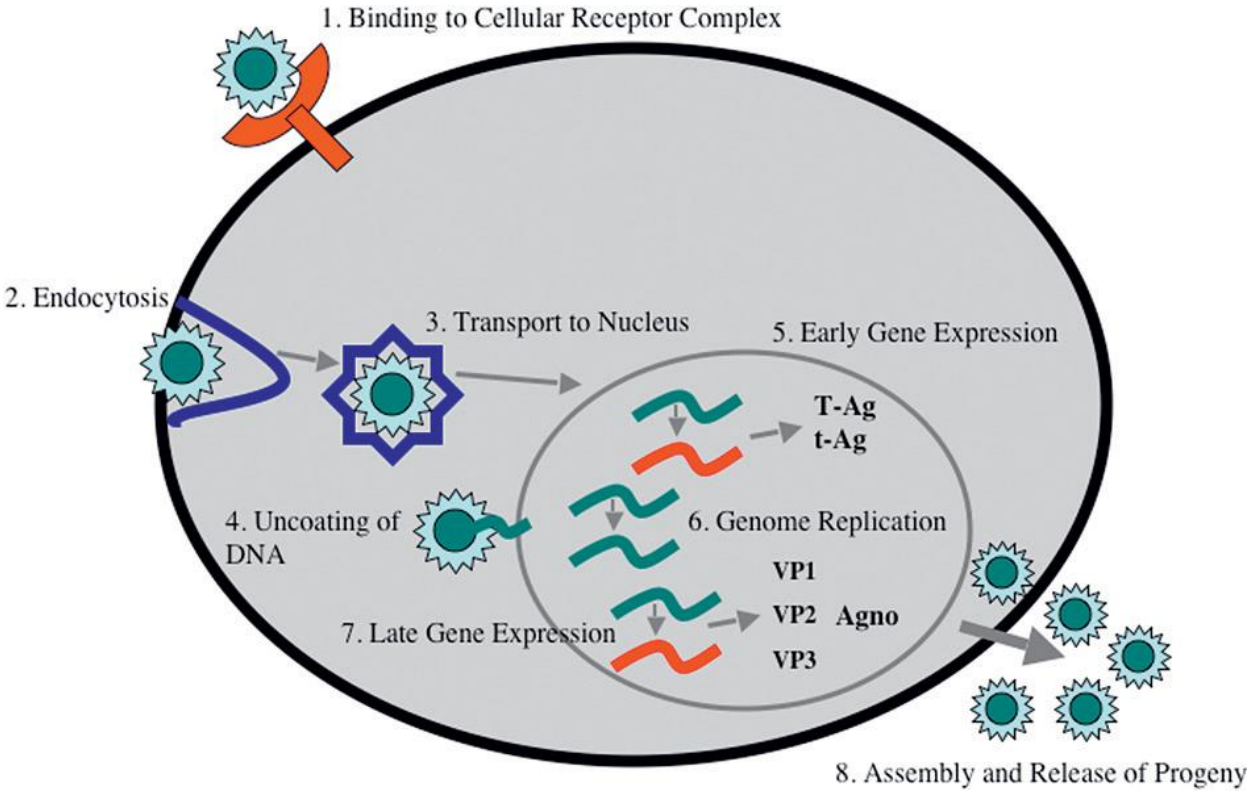

Figure 4. Life cycle of JCPyV: *[1]* The lifecycle starts when virus attaches to a cellular receptor complex. *[2]* Following this initial interaction, virus internalization into the cytosol happens via clatherin-dependent endocytosis. *[3]* Nuclear transport occurs where *[4]* uncoating happens thereafter to expose the genome for *[5]* early gene expression. *[6]* Viral DNA synthesis precedes *[7]* late gene expression. Finally, new virions are *[8]* assembled, which are released, thus marking the successful completion of productive infection (60).

JCPyV persistence and latency

JCPyV is excreted in the urine of healthy persons and in patients with PML. JCPyV remains latent in the kidneys, lymph nodes, and bone marrow of healthy and immunosuppressed individuals without PML and, upon reactivation, can cause a lytic infection of oligodendrocytes in the brain, leading to PML (229). It has also been detected in renal tissue, including that of healthy persons (39). These data suggest that the kidney serves as a site of latent infection, but the mechanisms and/or biochemical events that allow this are unclear (203). Bone marrow is another possible site for JCPyV latency. Susceptibility of infection has been demonstrated in both a CD34+ hematopoietic progenitor cell line, KG-1, and in primary cells (167). In addition, a current study by Tan *et al*. (229) suggests that JCPyV can spread throughout the body in immunosuppressed and immunocompetent individuals alike and that it is present in the brains of individuals without PML (Fig.5). The ability for JCPyV to persist for life in an infected host is a common characteristic of many DNA viruses, including herpesviruses, adenoviruses, and other polyomaviruses (20, 107). In a persistent infection, viral replication is kept in check by the host's immune system resulting in either a low or absent replication state. In doing so the virus has developed mechanisms to evade the immune system, allowing it to coexist with the host.

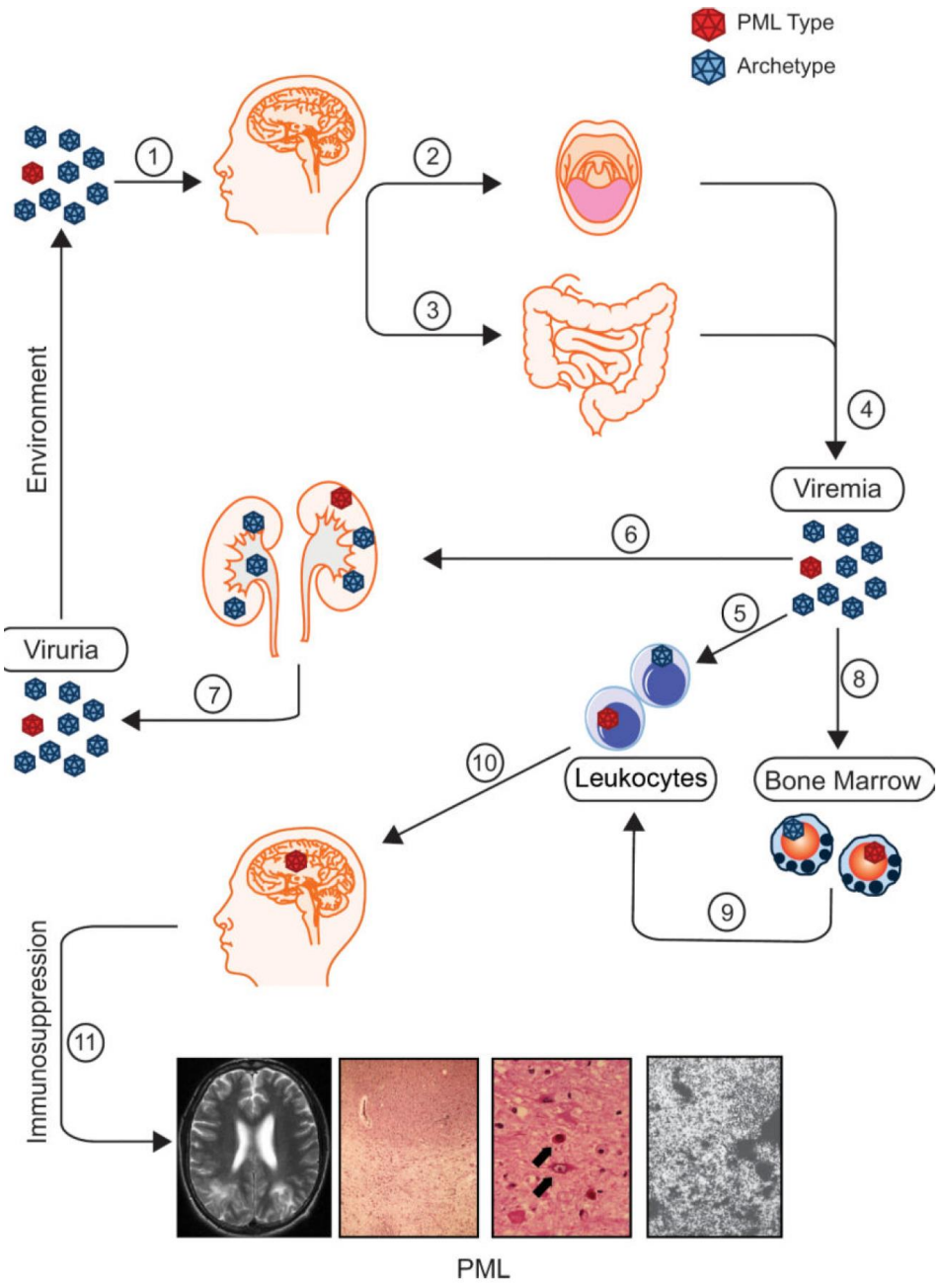

Figure 5. JCPyV dissemination and pathogenesis of PML: The events thought to occur during the JCPyV life cycle and pathogenesis of PML are shown with the common nonpathogenic events labeled in blue and the rare pathogenic events in red. Virions are thought to be transmitted through sewage contaminated material by inhalation or ingestion via the mouth and nose, *[1]* top right-hand corner, of predominantly archetype JCPyV (blue), but occasionally the neurotropic form (red). *[2]* It is thought to enter the bloodstream through either

the epithelium of the tonsils and the upper respiratory tract or *[3]* the gastrointestinal tract *[4]* to establish primary viremia . Currently, the nature of primary JCPyV viremia is not well understood, but *[5]* it has been suggested that virus may exist as free virions and/or as white blood cell-associated virus . *[6]* Virus is thought to spread to the kidney and other organs via a hematogenous route. *[7]* In the kidney, JCPyV can replicate sporadically and at low levels in the epithelium of the kidney tubules, from where it can shed from the apical face of the kidney epithelium. This shedding can lead to viruria and transmission of infectious virions via urine, completing the JCPyV lifecycle. *[8]* It is thought that virus can also spread to the bone marrow, where it has been speculated but not proven that neurotropic virus (red) can emerge by an unknown mechanism. *[9]* JCPyV may also undergo hematogenous spread from the bone marrow and possibly other locations in association with leukocytes, *[10]* including the brain, where neurotropic JCPyV DNA can be detected in healthy, immunocompetent individuals in the absence of expression of detectable levels of viral proteins. *[11]* Under immunosuppression , neurotropic JCPyV can become reactivated, undergo transcription and DNA replication, and spread to form microlesions, which can coalesce, increase in size, and result in PML. The pathological features of PML are shown in the bottom panels (left to right) T2-weighted MRI showing hyperintense signal abnormalities in the white matter of the parieto-occipital lobes, H and E staining demonstrating oligodendrocytes bearing nuclear inclusion bodies (upper arrow) and bizarre astrocytes (lower arrow), and TEM of PML tissue showing crystalline arrays of 45 nm viral particles within a nuclear inclusion body (256).

JCPyV reactivation and rearrangement

Although the prevalence of archetype JCPyV infection in the general population is high, the incidence of JCPyV PML in immunocompetent individuals is absent, indicating stringent immune mechanisms to prevent reactivation and disease in an immunocompetent host. The site and modality of JCPyV reactivation and rearrangement has yet to be conclusively described, although immunosuppression is a major component. The most likely hypothesis is that virus reactivation occurs in the periphery, where it infects circulating B lymphocytes, and through a Trojan horse mechanism these JCPyV-infected cells cross the blood-brain barrier to enter the central nervous system (CNS) where JCPyV infects astrocytes. JCPyV infection is usually restricted by the actions of the immune system, most notably cell-mediated immunity, where it is thought that there is an association between a defect in the generation of cytotoxic T cells and the reactivation of JCPyV from latency to cause PML (83, 128). Dormant JCPyV contains the archetype NCCR and has been described as predominantly associated with peripheral organs, including the tonsils, kidneys, spleen, and bone marrow. Whether archetype JCPyV is present in the CNS in immunocompetent individuals remains controversial (123, 185). Conversion of non-pathogenic archetype JCPyV to the neurotropic PML-causing JCPyV involves rearrangements in the NCCR, which regulates JCPyV transcription and DNA replication. However, the mechanism(s) by which JCPyV undergoes NCCR sequence alterations has yet to be described.

PML associated JCPyV VP1 mutations

Mutations in the major viral capsid protein 1 (VP1) of JCPyV have been suggested to favor the onset of PML. Several studies have reported the presence of several mutations in VP1 from JCPyV isolated from PML patients. These nonpolymorphic (i.e. JCPyV subtype-independent) PML-associated mutations or deletions of JCPyV VP1 include amino acids at positions 50, 51, 55, 60, 61, 122-125, 265, 267, 269, 271, and 283 (113, 262, 263). These studies suggest that the role of VP1 in PML pathogenesis might be attributed to its direct interaction with host immune responses, as well as cell attachment and viral entry via sialic acid receptors on susceptible cells (36, 135, 174). Thus, the virulence of PML associated JCPyV can be a result from changes in the affinity and specificity of the virus via its viral capsid for its cellular receptor(s) which directly affect viral infectivity and transmission.

PML, an always-fatal demyelinating disease of the CNS, is characterized by multiple foci of demyelination caused by the lytic infection of JCPyV infected oligodendrocytes (86, 116, 150, 194). PML was first described by Astrom *et al.* in 1958 (5), in a patient with lymphatic leukemia and a patient with Hodgkin's disease. Although viral etiology for PML was proposed by Cavanagh *et al.* in 1959 (29) and Zu Rhein and Chou demonstrated viral particles resembling Papovaviruses in 1965 via electron microscopy (265), it wasn't until 1971 that Padgett *et al.* cultivated JCPyV from the brain of a PML patient [1], where the virus was ultimately named after the initials of the patient. From 1958 to the 1980s, PML was primarily observed in patients treated with corticosteroids, other immunosuppressive drugs, and chemotherapy (134). It wasn't until the 1980s when PML became predominantly associated as a complication related to AIDS patients. Recently, the use of immunomodulatory and immunosuppressive drugs may increase the risk of development of PML in the setting in these immunosuppressive conditions (17).

Clinical and pathological features of PML

Primary JCPyV infection is typically subclinical (182), PML develops only in individuals with severely impaired immune systems, such as AIDS patients (117). PML has most often presented as an opportunistic infection in HIV patients with lymphopenia but recently it has been seen in patients treated with immunosuppressive drugs (22), including natalizumab, a monoclonal antibody (mAb) used to treat multiple sclerosis (MS) .

The onset of PML is insidious, but in the absence of treatment, disease progression is usually rapid, with death ensuing in 3 to 6 months after diagnosis. The clinical features of PML vary according to the localization of the demyelinating lesion and are non-specific. The periventricular and sub-cortical regions of the parieto-occipital and frontal lobes of the brain are the most affected regions (254). Common presenting symptoms include cognitive deficits, gait disorders, limb weaknesses, speech disorders and visual impairments (14, 64, 65).The pathogenesis of PML can be divided into 3 phases, with the first phase being a primary clinically inapparent infection. In the second phase, it has been suggested that a persistent and latent peripheral infection occurs within the urinary tract, bone marrow, and probably the spleen (244). The third and final phase is probably induced by immunologic and molecular alterations of the viral NCCR (245) resulting in reactivation of archetype JCPyV to the virulent rearranged JCPyV. Although the rearrangement of the NCCR of archetype JCPyV is thought to be an important event in the pathogenesis of PML, little is known about what induces this rearrangement. In addition, it is not known whether JCPyV is present in a latent state in the brain or whether it enters only after reactivation has occurred elsewhere in the periphery.

The neuropathological hallmarks of PML consist of multifocal microscopic and macroscopic demyelinating lesions that tend to coalesce in the subcortical white matter near the gray-white matter junction. Oligodendrocytes sustain productive lytic infection and when infected with

JCPyV, their nuclei become enlarged and filled with eosinophilic inclusion bodies. In addition, bizarre astrocytes appear enlarged, with multiple or multilobate hyperchromatic nuclei, at times resembling neoplastic cells (44) (Fig.6 and 7).

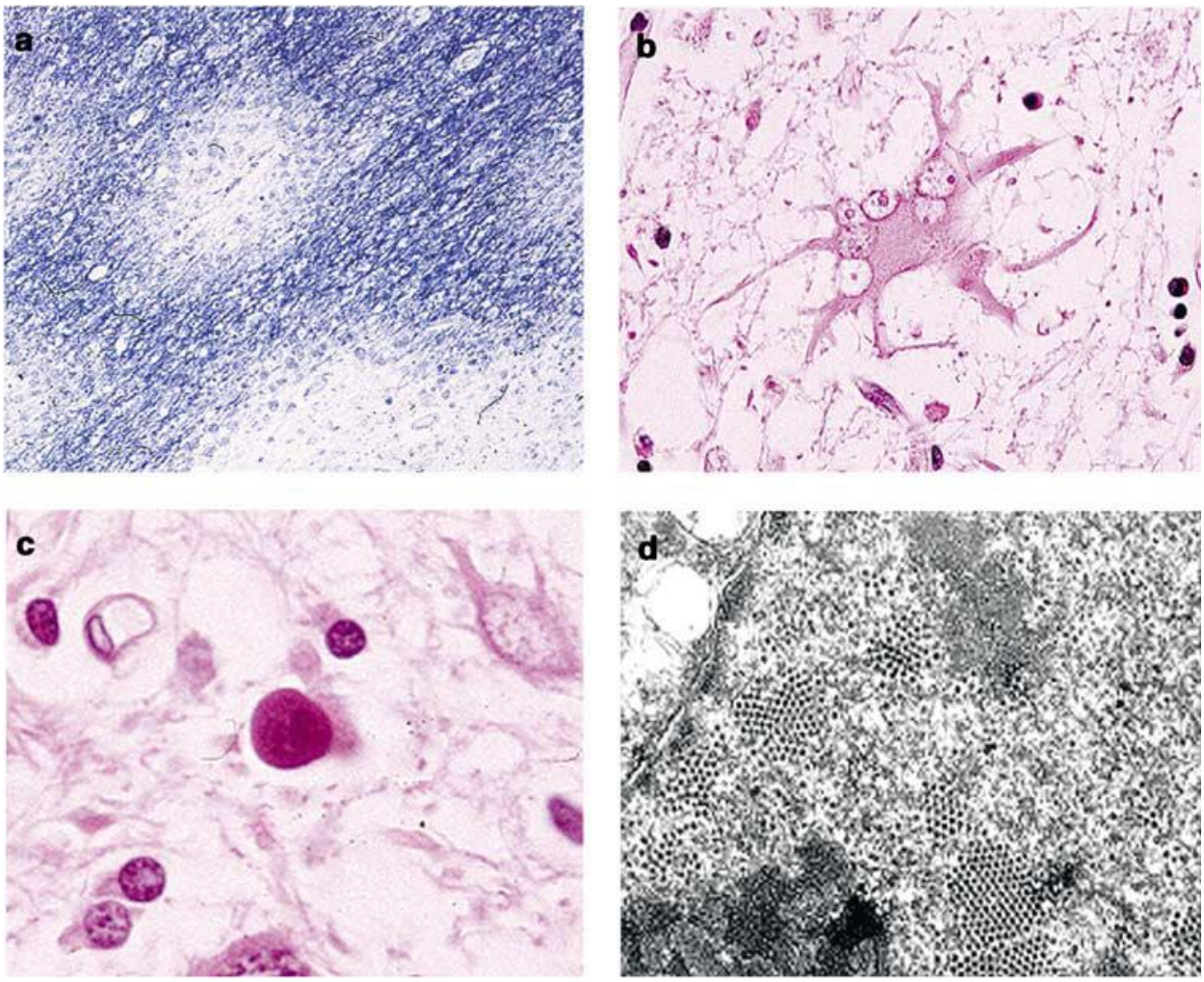

Figure 6. Histological features of PML: (a) Multiple areas of demyelination or plaques are observed at low magnification in paraffin-embedded sections of PML brain tissue stained with luxol fast blue. (b) Bizarre, transformed reactive astrocytes that may be multinucleated and resemble neoplastic cells are frequently observed in PML lesions. (c) Residual JCPyV-infected oligodendrocytes harboring intranuclear eosinophilic inclusion bodies can be seen with demyelinated plaques. (d) Electron microscopy of oligodendrocyte inclusions reveals the presence of 45 nm icosahedral viral particles in the nucleus consistent with JC virions (115).

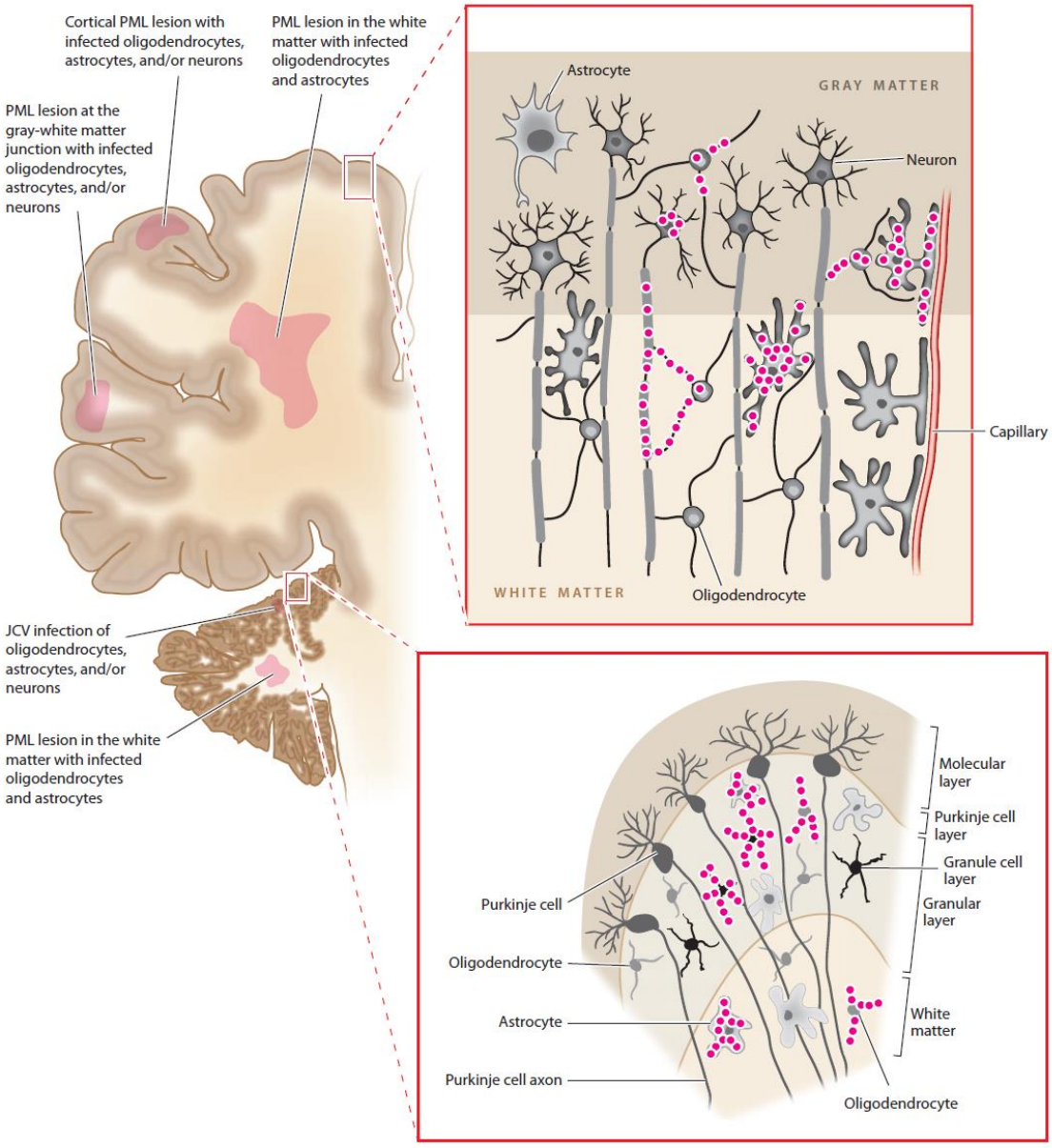

Figure 7. Brain lesions and cell types infected by JCPyV: The classical description of PML indicates demyelinating lesions of JCPyV infected oligodendrocytes and astrocytes located in the white matter of the cerebrum or the cerebellum. More recently, descriptions of lesions found in the cerebral cortex or at the gray matter-white matter junction with JCPyV-infected neurons have been described. When JCPyV infection occurs in granule cell neurons, cerebellar atrophy can occur (84).

PML in HIV/AIDS patients

Prior to the 1980s, PML was considered an extremely rare opportunistic infection; the incidence of PML was 0.15 cases per million population. However, the HIV pandemic led to a new subset of immunosuppressed individuals, resulting in a dramatic increase in the prevalence of PML to 0.6 cases per million (100). Currently, the most common predisposing factor for symptomatic JCPyV infection is HIV-induced immunodeficiency, with about one in 20 HIV-infected persons developing PML (8, 15, 16). The increased frequency of PML among patients with AIDS when compared to other immunocompromised patients suggests that the presence of HIV-1 in the brain of infected individuals is closely associated with the pathogenesis of AIDS-related PML (178). Thus, the significantly longer survival times reported for PML patients treated with highly active antiretroviral therapy (HAART) are primarily due to the successful reduction of HIV viral loads and resulting immunosuppression (244). The introduction of HAART has led to a significant prolonged median survival time, with AIDS patients living 4.5 years if their $CD4^+$ cell counts >100 cells/μL after diagnosis of PML. This is in contrast to the median survival time 3.4 years for those with $CD4^+$ cell counts <100 cells/μL after PML diagnosis (14). Although HIV accounts for an overwhelming majority of PML cases, in the order of 80%, there is increasing incidence of non-HIV related PML cases (98) (Fig.8).

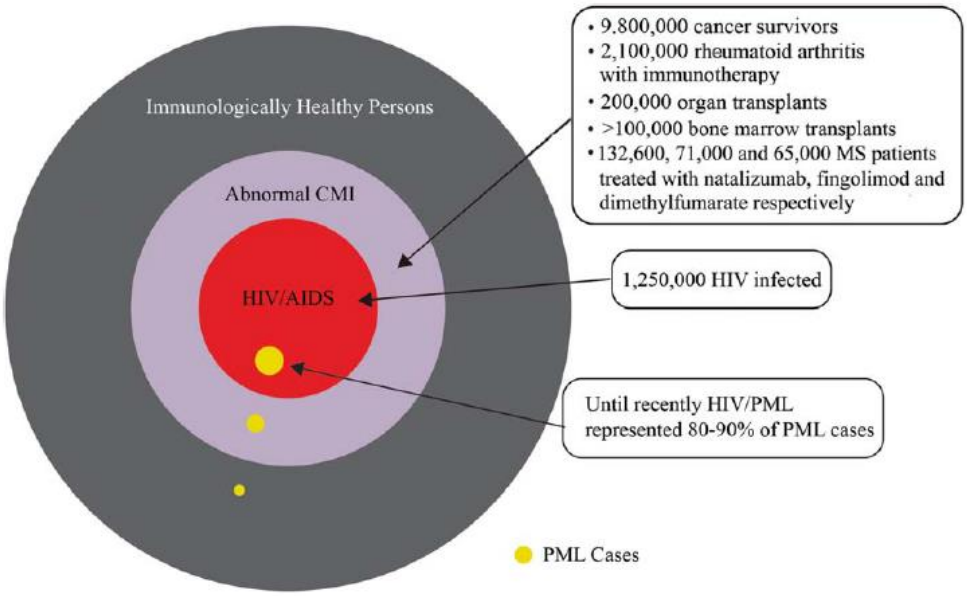

Figure 8. Occurrence of PML in the United States: The Venn diagram depicts the occurrence of PML, seen in the gold circles, in different populations of the United States. The outer circle represents the total population in 2012, which was 314 million individuals. The outer black ring represents the majority of healthy individuals in the population. This group of healthy individuals refer to those with no apparent case of immunosuppression and include the elderly, patients with chronic liver or kidney disease, and those with idiopathic or transient lymphocytopenia. The purple circle refers to a subpopulation of individuals who have impaired cell-mediated immunity (CMI), including, cancer survivors, bone marrow and solid transplant recipients, individuals suffering from rheumatoid arthritis treated with immunosuppressive agents like rituximab, and multiple sclerosis (MS) patients treated with natalizumab, fingolimod, or dimethyl fumurate. Lastly, the inner red circle represents individuals with impaired CMI due to HIV-1 infection/ AIDS, approximately 1.2 million individuals (256).

PML in HIV-uninfected individuals

The incidence of PML in HIV-uninfected individuals has increased with the broader use of immunosuppressive and immunomodulatory drugs used to treat an array of systemic and neurologic autoimmune disorders. These agents include, but are not limited to, chemotherapies, rheumatologic disease-modifying therapies, and multiple sclerosis (MS) treatments, which result in a decrease in immune surveillance of the CNS and therefore an increased risk of PML. PML has been associated with a variety of medications including alemtuzumab, belatacept, dimethyl fumurate, eculizumab, brentuximab, fingolimod, fludaribine, infliximab, leflunomide, mycophenolate mofetil, natalizumab, rituximab, among others.

In recent years, monoclonal antibodies (mAbs) have been used to treat a wide spectrum of immunological diseases. A resurgence of PML occurred in the 2000s as a result of the use of immunomodulatory compounds like the monoclonal antibodies natalizumab, efalizumab, and rituximab for the treatment of autoimmune diseases, including multiple sclerosis, Crohn's disease, severe forms of plaque psoriasis, hematologic malignancies, and rheumatoid arthritis (148). Some mAbs suppress the immune system and, as a result, predispose patients to PML (8). In 2005, PML developed in MS patients treated with the mAb natalizumab, trade name Tysabri (244), which is directed against the α4-integrin of the cell adhesion molecule family (8) (Fig.9).

In addition, hematologic malignancies, immunodeficiency disorders, idiopathic lymphopenia, and autoimmune rheumatologic disorders can lead to an increased risk of PML in the absence of pharmacologic therapies, which is likely due to the aberrant immune function associated with these conditions (210). Particular among autoimmune diseases, systemic lupus erythematosus (SLE) is associated with an increased risk of PML even in the absence of immunosuppression.

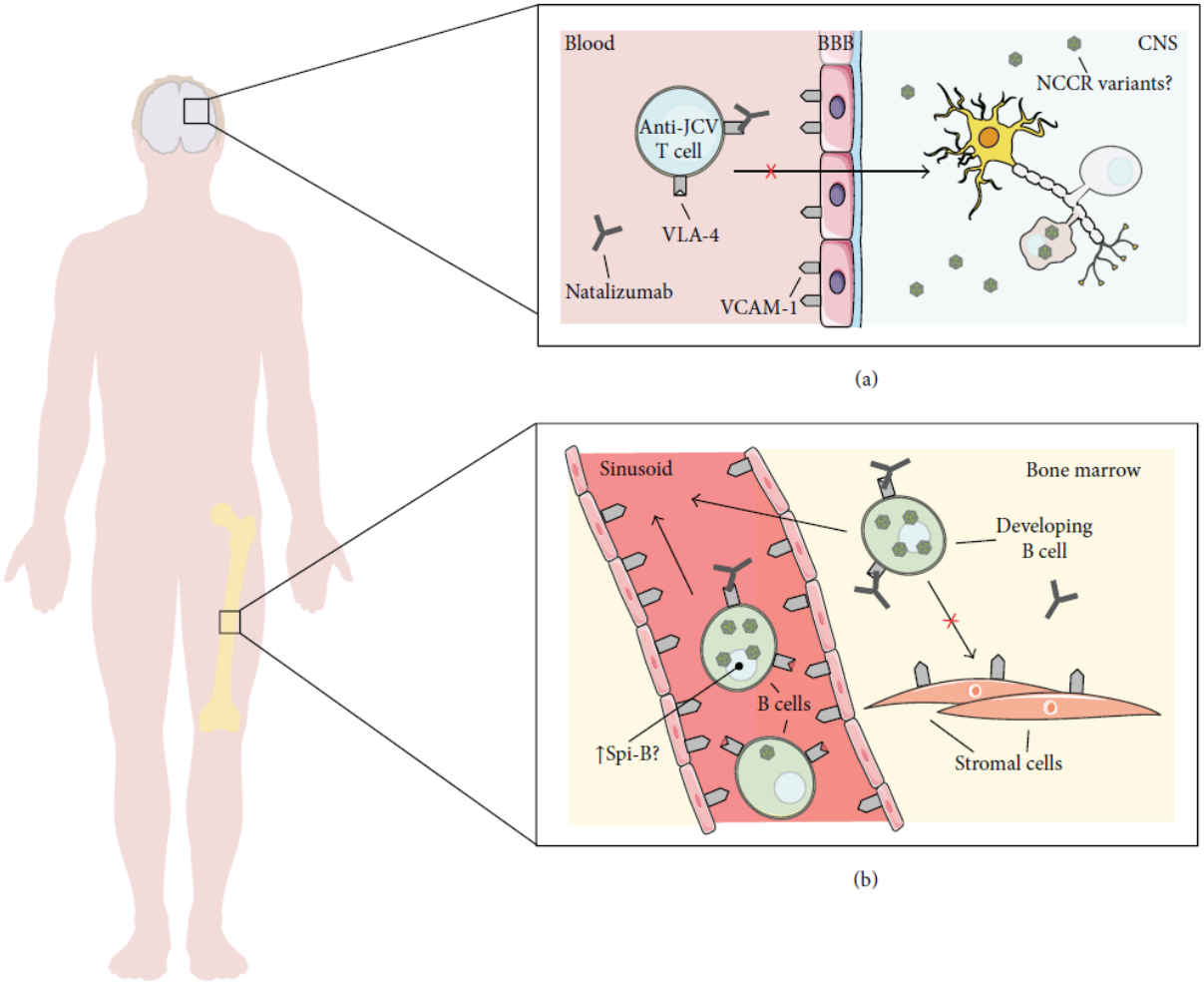

Figure 9. Natalizumab induced PML in MS patients: This figure summarizes three current hypotheses of how natalizumab may lead to PML. (a) Natalizumab may prevent entry of JCPyV-specific cytotoxic T cells into the brain, which are necessary to control latent JCPyV infected oligodendrocytes. (b) Natalizumab inhibits VLA-4-dependent homing and retention of lymphocytes in the bone marrow, a possible site of JCPyV latency, therefore resulting in an increase of JCPyV-infected peripheral leukocyte population. Lastly, it has been suggested that natalizumab-induced expression of Spi-B, a transcription factor associated with increased JCPyV transcription (54).

PML in immunologically normal individuals

PML is described as affecting individuals with severe immunosuppression such as HIV/AIDS, those receiving immunosuppressant therapy, those with hematological malignancy, and organ transplant recipients. Descriptions of PML in immunologically healthy individuals have been described in case reports, however, these individuals are described as being immunologically normal and immunocompetent in the context of either being HIV negative and/or using no medication or having significant medical history. In a recent review, immunologically healthy individuals are referred to those with no apparent case of immunosuppression and include the elderly, patients with chronic liver or kidney disease, and those with idiopathic or transient lymphocytopenia (Fig. 8) (256). This description therefore lumps individuals with causes of possible immune dysregulation as being immunologically healthy. This begs the question who is immunologically healthy?

This misinterpretation of immunologically healthy individuals becomes apparent when cases of PML are described in individuals that have a predisposing factor, such as older age, diabetes, and chronic infections, that may lead to immune dysregulation. Therefore, it is accurate to believe that all individuals presenting with PML are predisposed by some form of immune dysregulation. One such report, “Progressive Multifocal Leukoencephalopathy in a HIV Negative, Immunocompetent Patient,” (172) describes a 66-year-old male, with an undetectable JCPyV viral load in the CSF and a CD4+ >200, whom the authors define as being immunocompetent. However, upon further details it is noted that the patient had a history of HCV related cirrhosis and hepatocellular carcinoma. In another report, “Progressive Multifocal Leukoencephalopathy in an Immunocompetent Patient,” Aasly *et al*. describes a 72-year-old previously healthy woman who developed PML. The woman had a history of well-regulated hypertension and total alopecia at age 40 years with spontaneous improvements (110). There have been descriptions demonstrating associations between hypertension, proinflammatory

cytokines, and cells of the innate and adaptive immune systems (219). A series of cases by Gheuens *et al*. demonstrated that a certain degree of mild immunosuppression was present in 38 PML cases who were HIV-negative and free of malignancies (82). The associated conditions among these individuals included hepatic cirrhosis, chronic renal failure, dermatomyositis, pregnancy, and Alzheimer's disease.

Therefore, these case reports may not be the prototypical definition of immunosuppression usually associated with PML, but prove the point that immune dysregulation may warrant an environment conducive for JCPyV related PML. The underlying mechanism of JCPyV reactivation resulting in PML in an array of individuals makes it difficult to find one cohesive cause for PML pathogenesis. It may not be one mechanism of reactivation leading to PML but multiple roads diverging.

PML diagnosis and treatment

The diagnosis of PML can be thought of as a three-stage process that includes clinical suspicion, radiological identification, and confirmation by cerebrospinal fluid or tissue analysis (44). Clinical suspicion relies on the character and development of focal neurological symptoms and signs over time and disease susceptibility. Once PML is suspected, brain lesions are detected and characterized via MRI. In the case of PML, characteristic white-matter lesions in the brain areas associated with the clinical deficits can be visualized. Demyelinating lesions are usually hyperintense on T2-weighted and FLAIR MRI sequences, but hypointense on T1-weighted sequences, which indicate white matter destruction (Fig.10). Hypointense lesions help distinguish PML from other pathologies, primarily HIV-1 encephalopathy, which is characterized by diffuse central white-matter changes that are not detected on T1-weighted sequences.

Lastly, the laboratory methods used for a definitive PML diagnosis include detection of JCPyV DNA or proteins via *in situ* hybridization or immunohistochemistry on brain biopsy samples or by the detection of JCPyV DNA in CSF by PCR. Among patients with HIV-1 and neurological diseases not treated with HAART, the diagnostic sensitivity for PML with this technique was 72-92%, with a specificity of 92-100% (45). However, in recent times, it has been more common to see negative JCPyV PCR results in AIDS patients that have clinical and imaging presentations making these patients indistinguishable from those patients with PML. The decreased viral replication and clearance of JCPyV DNA from the CSF is thought to be associated with the immune restoration process as a result of antiretroviral therapy (41). As a result of this, the sensitivity of PCR testing for JCPyV DNA has dropped to 58% (8). Histologically, PML is characterized by a productive and lytic infection of both oligodendrocytes and astrocytes that lead to multiple areas of demyelination in the CNS. There may also be reactive gliosis and giant, bizarre astrocytes in affected areas (230). Currently, there are no specific antiviral drugs against JCPyV. Without a specific antiviral drug, the current treatment goal in PML is to restore the host-adaptive immune response to JCPyV for control of the infection. In HIV-positive patients, this goal is accomplished mainly by treatment of HAART. In HIV-negative patients, the main therapeutic objective is to reduce, if possible, immunosuppressive drugs, enabling the adaptive immune system to control the infection. However, in organ transplant recipients, decreasing these drugs increases the risk of graft rejection. Therefore, a better strategy might be to augment the cellular immune repose to JCPyV by use of immunotherapies such as dendritic cell vaccines (230).

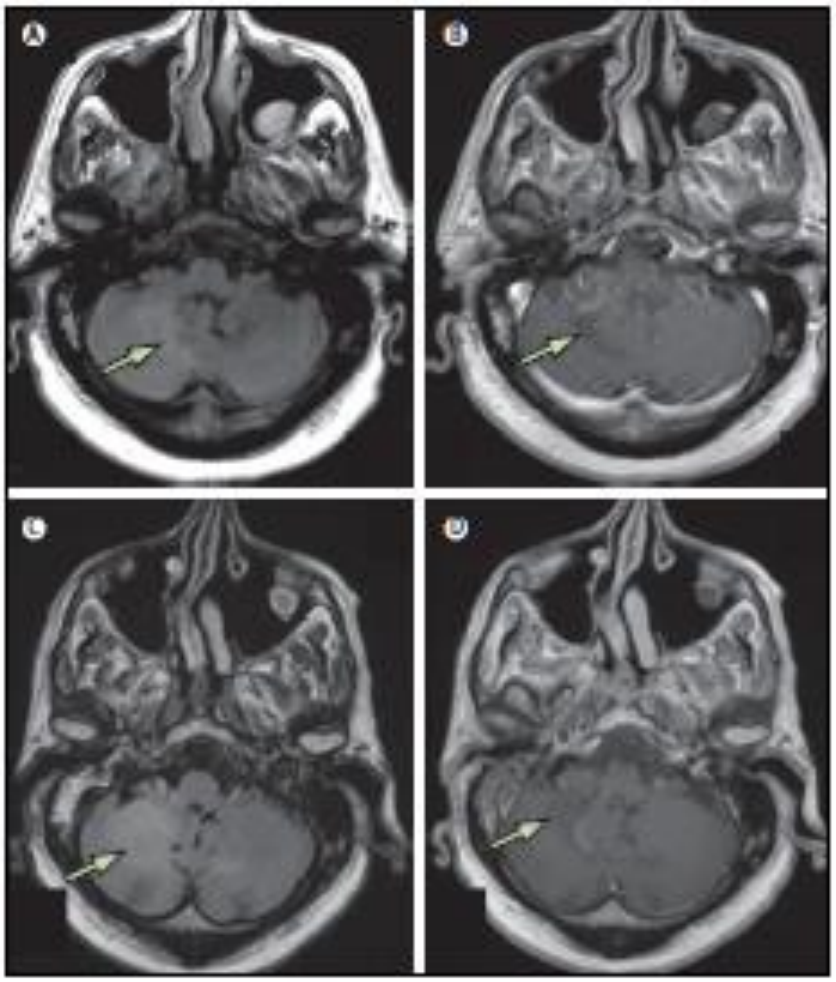

Figure 10. Cerebellar lesions in a patient with PML via MRI: An MRI scan of a lymphocytic leukaemia patient with classic PML. The patient was identified as JCPyV positive via PCR detection of JCPyV in the CSF. Lesions (arrows) were identified by fluid attenuated inversion recovery (FLAIR) (Fig 4A and 4C) and T1-weighted MRI (Fig 4B and 4D) (230).

PML-immune reconstitution inflammatory syndrome (IRIS)

IRIS is an inflammatory syndrome in response to clinically apparent or subclinical pathogens associated with the recovery of the immune system after a period of immunosuppression (Fig.11). In certain cases, a rapid global recovery of the immune system may not be favorable. So although a cellular immune response directed against JCPyV is beneficial in classic PML, PML-IRIS can be triggered if such a recovery of the immune system were to occur. HIV-1 associated PML-IRIS comprises of three elements. First, immune reconstitution, meaning a decrease of plasma HIV-1 RNA with or without an increase in $CD4^+$ T cells associated with the start of combined antiretroviral therapy. Second, tissue inflammation, and third, clinical disease or worsening that would not be expected from the natural course of the disease (77, 215). IRIS may occur during either of the two phases of immune restitution that occurs after the initiation of HAART (197). The first period of susceptibility occurs in the initial weeks when the increase in $CD4^+$ T cells is largely due to the redistribution of pre-existing memory T cells. The late phase is a direct result of the proliferation of naïve T cells, usually after 4-6 weeks but can be as long as 4 years after the initiation of HAART (103). In PML, IRIS occurs in two settings, the first known as paradoxical IRIS, where inflammation develops in relation to existing lesions as a result of the symptomatic disease being treated with combined antiretroviral therapy. The second setting, known as unmasking IRIS, happens when patients develop PML after the start of combined antiretroviral therapy and an inflammatory picture is found via MRI (76).

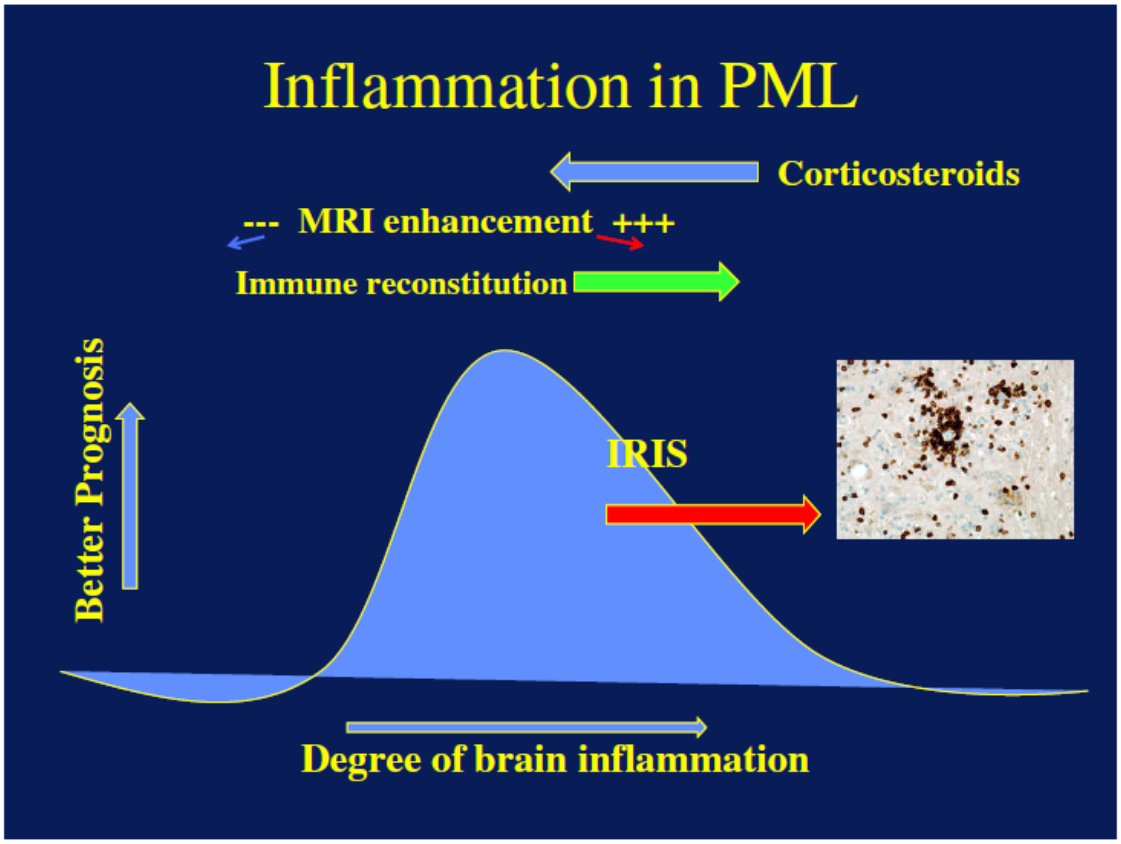

Figure 11. PML induced immune reconstitution inflammatory syndrome (IRIS):

Unregulated or prolonged immunosuppression can lead to poor clinical prognosis of PML. As the immune response increases, for example by weaning a patient off of immunosuppressive drugs or starting HAART, prognosis improves as JCPyV is controlled (x-axis to the right). At some point prognosis once again declines as pathological IRIS develops. Inset depicts marked CD3 infiltration into the brain of a patient with IRIS that would not be found in a severely immunocompromised individual developing PML. Increases in inflammation breaks down the blood-brain barrier during immune reconstitution. However, clinical intervention with corticosteroids can shift the inflammatory response to the left (47).

JCPyV animal models

Many studies have established the oncogenic potential of JCPyV in laboratory animals but demonstrating infection, pathogenesis, and PML has remained elusive. The major challenge in creating an animal model for JCPyV infection and disease is the inability of JCPyV to replicate in nonhuman cells. It has been demonstrated that in owl monkeys (*Aotus* trivirgatus) and squirrel monkey *(Saimiri sciureus*), JCPyV infection does not progress past the early phase of infection where only TAg is expressed with a lack in expression of capsid proteins and no viral DNA replication (251). Inoculation with JCPyV intracerebrally, subcutaneously, or intravenously in owl monkey and squirrel monkey models resulted in the development of astrocytoma, glioblastoma, and neuroblastoma (140, 141). Interestingly, juvenile owl monkeys inoculated intracerebrally with Mad-1 JCPyV remains the only report of infectious virus recovered from tissue or tumors of any experimental animal species inoculated with JCPyV (153). Similar results were demonstrated in rodent models, for instance, when newborn golden Syrian hamsters (*Mesocricetus auratus*) were inoculated subcutaneously and intracerebrally with JCPyV multiple brain tumors resulted (243, 264). Recently, a novel mouse model engrafted with human lymphocytes and thymus, designated humanized NOD/SCID/IL-2-Rg (null) mice, has been described (226). Mice inoculated with JCPyV remained asymptomatic, however, JCPyV DNA was occasionally detected in the blood and urine of infected animals. Interestingly, mice generated both humoral and cellular immune responses in conjunction with the expression of the immune exhaustion marker, PD-1, consistent with response to infection (226). Although humanized mice represent a novel animal model to study the interactions of JCPyV with the immune system, this model along with the previously described animal models does not embody a working model to study PML pathogenesis. A summary by Khalili *et al*. of previously studied JCPyV animal models are described in Table 2 (251).

Table 2. JCPyV animal models

Species	Agent	Introduction	Outcome	NCCR	References
Owl monkey/ Squirrel monkey	Purified JCPyV	Intracerebral injection	Astrocytoma	Mad-1	London *et al.* (1978) (140); Houff *et al*. (1983) (101); London *et al*. (1983) (141)
Owl monkey	Purified JCPyV	Intracerebral injection	Astrocytoma	Mad-1	Major *et al.* (1987) (153)
Syrian golden hamster	Purified JCPyV	Intracerebral injection	Glioma	Mad-1	Walker *et al.* (1973) (243)
Syrian golden hamster	Purified JCPyV	Intracerebral injection	Medulloblastoma	Mad-4	Zu Rhein and Varakis (1979) (264)
Mice	JCPyV TAg	Transgenic	Adrenal Neuroblastoma	Mad-1	Small *et al.* (1986) (220)
Mice	JCPyV TAg	Transgenic	CNS Dysmyelination	Mad-1	Small *et al.* (1986) (221); Trapp *et al.* (1988) (235); Hass *et al.* (1994) (91)
Mice	Polyoma TAg	Transgenic	CNS Dysmyelination	Murine PyV	Baron-van Evercooren *et al.* (1992) (9)
Mice	JCPyV TAg	Transgenic	Medulloblastoma/ PNET	Archetype (CY)	Krynska *et al.* (1999) (130)
Mice	JCPyV TAg	Transgenic	MPNST	Mad-4	Shollar *et al.* (2004) (217)
Engrafted NOD/SCID/IL-2-Rg (null) mice	Purified JCPyV	Intraperitoneal injection	Anti-JCPyV immune response	Mad-4 or CY	Tan *et al.* (2013) (226)
SHIV infected Rhesus monkeys	SV40	Intravenous injection	Meningo-encephalitis and demyelination	SV40	Axthelm *et al.* (2004) (7)
Nude mice	JCV-infected human cells	Intracerebral injection	Persistence of infected cells	Mad-1	Matoba *et al.* (2008) (160)
Engrafted $Rag2^{-/-}$ $Mbp^{shi/Shi}$ mice	Purified JCPyV	Intracerebral injection	Demyelination	Mad-1	Kondo *et al.* (2014) (126)

(251) T Antigen (TAg), Primitive neuroectoddermal tumor (PNET), Malignant peripheral nerve sheath tumor (MPNST)

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CHAPTER 2

DISSERTATION SCOPE

Background for research question

Understanding the mechanisms underlying the development of PML has been hampered by the inability to conclusively delineate the sites of JCPyV latency and reactivation in humans, the inability to demonstrate rearrangement of archetype JCPyV in an *in vitro* replication model, and the absence of an *in vivo* animal model to study JCPyV pathogenesis. To date, no experimental studies have been conducted to demonstrate the infection of urine-derived archetype JCPyV in primary renal proximal tubule epithelial (RPTE) cells, the reactivation and/or rearrangement of archetype JCPyV, and the infection of archetype JCPyV in an *in vivo* animal model. Thus, there is a ***gap*** in our understanding of the primary sites of initial infection, the events responsible for the reactivation and rearrangement of archetype JCPyV into the pathogenic rearranged form that causes PML, and a suitable animal model to study the pathogenesis of JCPyV.

Long-Term Goal, Objective, and Hypothesis

Our ***long-term goal*** is to delineate the natural history of archetype JCPyV infection, reactivation, and rearrangement for evidence-based approaches to improve treatment for PML. The ***objective*** of this study is to understand the cellular and molecular events leading to JCPyV infection, rearrangement, and/or reactivation. The ***aims*** of the proposed research are to determine the tropism of urine-derived archetype JCPyV in primary RPTE cells, to delineate the importance of genomic alterations in the pathogenesis of PML, to establish an *in vitro* model of JCPyV rearrangement, and to establish an *in vivo* JCPyV animal model. We ***hypothesize*** that i) urine-derived archetype JCPyV will be able to infect RPTE cells, ii) the presence of TAg will induce JCPyV NCCR rearrangements in an *in vitro* system, iii) alterations to the viral capsid will result in altered JCPyV replication kinetics and iv) archetype and rearranged JCPyV can infect NOD *scid* gamma (NSG) mice.

Specific Aims

Specific Aim 1: Investigate archetype JCPyV rearrangement and cellular tropism using brain and kidney *in vitro* model system.

Specific Aim 1a: Investigate archetype JCPyV infection and replication in primary RPTE cells.

Specific Aim 1b: Demonstrate rearrangement of archetype JCPyV in a linear reinfection model using COS-7, RPTE, and primary human brain microvascular endothelial (HBMVE) cells.

Specific Aim 1c: Determine the cellular tropism and replication kinetics of urine-derived rearranged archetype JCPyV replication using RPTE, HBMVE, human brain cortical astrocytes (HBCA) and primary human fetal glial (PHFG) cells as *in vitro* model systems.

Hypothesis: We *hypothesize* that propagated urine-derived archetype JCPyV will infect primary RPTE cells. Furthermore, sequential reinfection and passaging of urine-derived archetype JCPyV in COS-7 cells constitutively expressing TAg and/or in primary cells will result in changes to the NCCR leading to altered cellular tropism.

Approach: The susceptibility of primary RPTE cells to archetype JCPyV infection will be monitored by detection and analysis of JCPyV DNA, RNA, protein, and infectious virions. Quantitative analysis of JCPyV genome copies and RNA transcripts will be conducted using qPCR and qRT-PCR of JCPyV TAg and VP1 from days 1 to 20 after infection, as previously described (32). JCPyV TAg protein will be detected by immunoprecipitation/ western blot (IP/WB) and JCPyV VP1 protein by immunofluorescence assay (IFA) from archetype JCPyV-infected primary RPTE cells at 10 and 15 days after infection (35). Confirmation of the presence of viral particles will be demonstrated via transmission electron microscopy (TEM). NCCR sequence analysis will be conducted at days 0, 5, 10, 15, 20, 25, 30 and 35, of archetype

JCPyV-derived from infected RPTE cells. To demonstrate the production of infectious archetype JCPyV virions, naïve RPTE cells will be infected with virus isolated from previously infected RPTE cells collected at day 35 after infection. After archetype JCPyV infection of COS-7 cells, cells will be passaged every 10 days and one half of the infected cells will be kept for continuous growth, and remaining cells will be used for DNA extraction. After archetype JCPyV infection of primary RPTE and HBMVE cells, a portion of infected cells will be used for DNA extraction, with the remainder of the cells will be lysed and used to reinfect naïve cells. Rearrangement of archetype JCPyV will be monitored every 10 days by NCCR PCR and sequence analysis.

Specific Aim 2: Investigate the role of JCPyV VP1 alterations in viral replication and JCPyV pathogenesis

> **Specific Aim 2a:** Determine if archetype JCPyV +/- VP1 mutations alter viral DNA replication, infectious virus production, and non-coding control region (NCCR) rearrangement following transfection into HBMVE cells.
>
> **Specific Aim 2b:** Demonstrate that replication activity of transfected JCPyV archetype plasmids is comparable to infection in HBMVE cells.

Hypothesis: We *hypothesize* that VP1 alterations in JCPyV will result in an increase in virus replication, an increase in virus production, and NCCR rearrangement following transfection into HBMVE cells.

Approach: Primary HBMVE cells will be transfected with 25 ng of either parental constructs of archetype JCPyV (CY), rearranged JCPyV (Mad1 or M1), or hybrid JCPyV (CYrM1c), which contains an archetype NCCR in the backbone of rearranged JCPyV (Mad1) coding region. HBMVE cells will also be transfected with 25 ng of either CYrM1c constructs with the VP1

mutations CYrM1c-S267F or CYrM1c-L55F. Cells will be harvested at 4 hours and at days 3, 5, 10 and 15 after transfection, and DNA and RNA will be extracted to analyze viral replication kinetics using qPCR and qRT-PCR for JCPyV early and late genes, TAg and VP1, respectively. In addition, primary human fetal glial (PHFG) cells will be transfected with 25 ng of M1, M1-L55F, or M1-S267F, amplified via VP1 PCR, and sequenced. Lysate from PHFG transfected either with M1, M1-L55F, or M1-S267F will be sonicated, titered, and used to reinfect PHFG cells to demonstrate production of infectious virions. Lastly, COS-7 cells will be transfected with 25 ng of CY to propagate infectious virus used to infect HBMVE cells. VP1 and NCCR sequence analysis will be conducted after each experiment.

Specific Aim 3: Determine the susceptibility of humanized NSG mice to archetype and rearranged JCPyV infection.

Hypothesis: Based on published literature showing human specific pathogen infection in humanized NSG mice (85, 209, 226) and JCPyV infection in human B cells (31, 166, 247), we *hypothesize* that JCPyV can infect humanized NSG mice.

Approach: Prior to infection, human immune cells engrafted in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice will be confirmed by flow cytometry analysis. NSG mice will then be infected with either archetype JCPyV or Mad-1 rearranged JCPyV by intravenous injection. Blood and urine will be collected at 3, 5, 7, 14, 21, and 28 days after infection. JCPyV viral DNA and TAg protein in NSG mice will be detected by quantitative PCR (qPCR) and flow cytometry.

Significance

Even with the advent of HAART, the incidence of PML has not changed. PML remains one of the important causes of mortality and morbidity among HIV/AIDS patients, with approximately 4% of AIDS-related patients developing PML. In addition, the incidence of PML in HIV-uninfected individuals has increased with the broader use of immunosuppressive and immunomodulatory drugs used to treat an array of systemic and neurologic autoimmune disorders. These agents include, but are not limited to, chemotherapies, rheumatologic disease-modifying therapies, and multiple sclerosis (MS) treatments, which result in a decrease in immune surveillance of the CNS and therefore increased risk of PML in JCPyV infected individuals. Therefore, the lack of an archetype JCPyV replication, reactivation, and rearrangement model has hindered the understanding of mechanisms underlying the development of JCPyV pathogenesis and progression to PML. Unfortunately, there are no preventive or therapeutic options available to manage PML patients. Thus, the proposed study is significant in that it will utilize primary cells to meticulously delineate steps involved in primary archetype JCPyV pathogenesis and the understanding of such mechanisms may assist in developing preventative or therapeutic interventions for this incurable disease.

PML is a subacute demyelinating disease of the CNS caused by the ubiquitous polyomavirus JC (JCPyV) (80, 195). The onset of PML is insidious, first presenting with neuropsychological deficits. The natural disease progression is usually rapid, with death ensuing in 3 to 6 months after diagnosis. The neuropathological hallmarks of PML consist of multifocal microscopic and macroscopic demyelinating lesions typically in the subcortical white matter near the gray-white matter junction. Ultrastructural examination reveals nuclei of infected oligodendrocytes packed with electron-dense JCPyV particles, measuring approximately 40 nm in diameter (196). PML was originally recognized as a rare complication of hematological malignancies or systemic

inflammatory disorders, however, a dramatic 50-fold increase in the incidence in the last thirty years occurred as a result of the HIV/AIDS epidemic (12). AIDS is the most frequent condition associated with PML (132), with approximately 6% of patients developing AIDS related PML (3, 164). Moreover, a recent report on 151 brain pathology confirmed that in the post-HAART era cases of PML remained unchanged (132). A resurgence of PML occurred in the 2000s as a result of the use of immunomodulatory compounds like the monoclonal antibodies natalizumab, efalizumab, and rituximab for the treatment of the autoimmune conditions such as, multiple sclerosis, Crohn's disease, severe forms of plaque psoriasis, hematologic malignancies, and rheumatoid arthritis (148).

JCPyV, a member of the genus, *Orthopolyomavirus*, has a naked icosahedral capsid and a circular double-stranded DNA genome of about 5.1 kb (118, 146). The viral genome is functionally divided into an early region (2.4 kb) encoding large and small T proteins along with the recently described T' proteins generated by alternative splicing of the early mRNA; a late region (2.3 kb) encoding viral capsid proteins VP1, VP2 and VP3, the accessory agnoprotein; and a non-coding regulatory region (0.4 kb) encompassing the noncoding control region (NCCR). Based upon the structure of the NCCR, two types of JCPyV have been identified: the archetypal form, which is predominantly detected in kidney and urine and the rearranged form which is predominantly detected in brain, tonsil and lymphocytes (81, 213). Archetype JCPyV is detected in the urine of people with and without PML, and its NCCR consists of 6 regions designated A-F. Conversely, the regulator region of JCPyV isolated from PML patients display rearrangements, with deletions, duplications, tandem repeats, and insertions. It is thought that all other rearranged forms of JCPyV arise from the archetype form, and most likely arise during immunosuppression. Serological data indicate that JCPyV infection usually occurs during childhood and is typically subclinical (182). Asymptomatic JCPyV infection occurs in 60 to 80% of healthy individuals. The route of JCPyV transmission and the primary sites of replication are

unknown, although, viruria is common, and transmission via urine to oral/respiratory route and primary replication in tonsillar tissue has been proposed (166, 168, 213, 241). Virus-infected lymphocytes or cell-free virus presumably spread by the hematogenous route from the primary site to secondary sites, such as kidneys, lymphoid tissues and brain, to establish focal areas of infection or persistence (56, 166, 168, 176, 233, 249). PCR analyses have suggested that JCPyV may persist in brain, tonsils and lymphocytes of individuals with and without PML (56, 168, 175, 176, 233, 249), and PML might arise from reactivation of JCPyV.

The host cell range of archetype JCPyV is strictly restricted in cultured cells, where researchers have demonstrated poor to moderate replication of archetype JCPyV in transformed cell lines, such as PHFG cells transformed with an origin-defective mutant of simian virus 40 (SV40) (POJ-19) and simian kidney cells transformed with an origin-defective mutant of SV40 (COS-7) cells, respectively (50, 95). In vitro data indicates that various archetype JCPyV DNA clones can initiate efficient virus replication with the conservation of the NCCR after transfection in COS-7 cells (95). In contrast, it has been demonstrated that rearranged Mad-1 JCPyV can efficiently replicate in primary cells, including PHFG and HBMVE cells (35). Therefore, it has yet to be determined if archetype JCPyV can infect and replicate in primary RPTE cells.

Cell type specificity of JCPyV within human cells occurs at the transcriptional level. Regulation of transcription is dependent on the sequence of the NCCR, as well as the availability of host transcription factors, which are the determining factor in both the start sites of early transcription, as well as the quantity of T antigen produced (70). Unlike other human DNA viruses, such as herpesviruses, JCPyV does not bring transcriptional activating proteins into newly infected cells. Although host cell factors are the determining factor in directing early

transcription, the exact profile of transcription factors involved in reactivation and rearrangement remain elusive.

Interestingly, *in vivo* studies have confirmed the oncogenic potential of JCPyV but due to JCPyV's strict host tropism, demonstrating infection, pathogenesis, and PML in animals has been limited. Recently, a novel mouse model engrafted with human lymphocytes, designated humanized NOD/SCID/IL-2-Rg (null) mice has been described (226). However, infection using urine-derived archetype JCPyV in this model system has yet to be described.

Innovation

Previous studies have either used a transfection based JCPyV method or utilized transformed cell lines to try to address questions regarding JCPyV replication. The proposed research utilizes *in vitro* primary HBMVE and RPTE cells, which have never been described in archetype JCPyV infection. This study aims to identify the differences in the host cell tropism of archetype JCPyV, to establish an *in vitro* model to study genomic alterations of archetype JCPyV, and develop an *in vivo* animal model to study JCPyV infection. Furthermore, for the first time, we have demonstrated *in vitro* rearrangement of archetype JCPyV. Utilizing this rearranged strain of JCPyV we will be able to delineate the events that have led to this rearrangement by addressing the transcription factor profile and cellular tropism. By utilizing these primary cells, future studies will be done to identify transcription factors involved in the reactivation and rearrangement of urine-derived archetype JCPyV. This study will lay the foundation to further understand how host cell tropism and transcription factors play a role in the latency, productive infection, reactivation, and rearrangement of JCPyV, which will ***impact*** the development of much-needed therapeutic interventions for PML.

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CHAPTER 3

TROPISM AND REARRANGEMENT OF ARCHETYPE HUMAN POLYOMAVIRUS JC

Tropism and rearrangement of archetype human polyomavirus JC

Running title: Characterization of archetype JC virus

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Abstract

Background: The human polyomavirus JC (JCPyV) is the causative agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). While the archetypal form of the virus is ubiquitous in the healthy human population, it is the rearranged form that is responsible for PML. The archetype form of JCPyV has a conserved noncoding control region (NCCR) that is defined by six designated blocks, A-F. However, the rearranged form has deletions and/or duplications in its NCCR. Although it has been established that the rearranged form of JCPyV is pathogenic, the events leading to the reactivation and/or rearrangement in its NCCR have yet to be determined. Thus, the lack of an archetype JCPyV replication model has hindered the understanding of mechanisms underlying the development of PML pathogenesis.

Methods: JCPyV isolated from urine was purified using sucrose gradient. JCPyV replication kinetics conducted in primary renal proximal tubule epithelial (RPTE), human brain microvascular endothelial (HBMVE), human brain cortical astrocytes (HBCA) and primary human fetal glial (PHFG) cells was characterized using quantitative PCR (qPCR), reverse transcriptase-PCR (qRT-PCR), hemagglutination assay (HA), immunofluorescence assay (IFA), immunoprecipitation (IP)/western blot (WB) and transmission electron microscopy (TEM). COS-7, HBMVE, and RPTE cells were infected with urine-derived archetype JCV and passaged every 10 days. Cell lysates were collected for DNA and RNA analysis and for reinfection. Characterization of the novel rearranged virus isolated from infected COS-7 cells at day 645 was performed by infecting HBMVE, HBCA, and RPTE cells. Following infection, DNA and RNA was collected at designated time points over the course of 35 days, whereby the replication kinetics were observed by qPCR and qRT-PCR. Lastly, predictive bioinformatics analysis determined transcription factor binding motifs present in urine-derived archetype JCPyV and D645 rearranged JCPyV as compared to CY and Mad-1 variant.

Results: JCPyV TAg protein was detected in RPTE and HBMVE cells by IP/WB and JCPyV VP1 protein by IFA. Viral particles within the nucleus of RPTE and HBMVE cells were detected by TEM. Re-infection of naïve RPTE and HBMVE cells with lysates from archetype infected RPTE and HBMVE cells demonstrated an exponential increase of DNA and RNA 15 days after infection. Sequence analysis of infected HBMVE, RPTE, HBCA, and PHFG cells demonstrated no alterations in the genome of archetype JCPyV. Interestingly, rearrangement of urine-derived archetype JCPyV NCCR occurred *in vitro* at 645 days after infection in COS-7 cells. At 645 days after infection in COS-7 cells, one base pair substitution in block A, one base pair substitution in block B, an 8 base pair insertion in block C, and 5 base pair deletion in block F were observed in the NCCR, with no changes to VP1. Characterization of the replication kinetics of day 645 rearranged virus demonstrated limited replication in HBMVE and RPTE cells, and non-productive replication in HBCA cells. Predictive bioinformatic analysis reveals distinct transcription factor binding sites that may give insight into differences in JCPyV replication.

Conclusions: These data demonstrate infection and efficient replication of archetype JCPyV in RPTE and HBMVE cells, and limited or no replication in HBCA and PHFG cells. To our knowledge, this is the first time demonstrating the ability for urine-derived archetype JCPyV to rearrange *in vitro* and to be infectious in naïve primary cells. By identifying the differences in the cellular tropism of urine-derived archetype JCPyV, D645 rearranged JCPyV, CY, and Mad-1 variants and profiling transcription factors important in the replication, rearrangement, and/or reactivation of these variants, this study will therefore give insight on cellular conditions involved in urine-derived archetype JCPyV pathogenesis.

Introduction

The human polyomavirus JC (JCPyV) belongs to the *Polyomaviridae* family and has a naked icosahedral capsid with a circular double-stranded DNA genome that is approximately 5.1-kb in length and consists of three different regions: the noncoding control region (NCCR), the early coding region, and the late coding region. The early transcriptional unit encodes the small and large T antigens, whereas the late transcriptional unit encodes the agnoprotein and the viral structural proteins VP1, 2, and 3.

Based upon the structure of the NCCR, two types of JCPyV have been identified in humans, the archetypal form and the rearranged form. Archetype JCPyV is found in the urine of healthy people, as well as those affected with PML (260) and is the form that is thought to circulate in the human population. Minor sequence variation exists in the genomes of independent isolates of archetype JCPyV (259, 260). The rearranged form of JCPyV has deletions and/or duplications in the NCCR sequence and is thought to evolve from archetype JCPyV (260). First isolated in 1971, the rearranged form of JCPyV has been associated with PML (183). PML is the only known human viral demyelinating disorder and is characterized by multiple foci of demyelination caused by the lytic infection of oligodendrocytes by JCPyV. Once considered to be rare, a resurgence of PML in the last 30 years has been attributed to HIV/AIDS and more recently to the use of monoclonal antibodies to treat multiple sclerosis and autoimmune conditions (28). Although it has been suggested that the rearrangement of the NCCR is critical in the development of PML among immunocompromised patients, the exact mechanism of NCCR rearrangement has not been clearly defined (150). It has been suggested that the rearranged form may be generated during virus replication, yielding a new, potentially more active form of the virus (49). The regulatory region of JCPyV isolated from the CSF and brain of PML patients shows rearrangements, including duplications, tandem repeats, insertions and

deletions. The rearranged form has been reported in the tonsils and lymphocytes of people with and without PML (213). However, NCCR rearrangement of archetype JCPyV has yet to be demonstrated *in vitro*.

Using various molecular techniques, archetype JCPyV has been detected in various human organs and tissues, including kidney (39, 123), gastrointestinal tract (193, 211), tonsil (88, 112) and bone marrow (159). However, the exact site(s) of archetype JCPyV infection and replication and the cell types that harbor latent archetype JCPyV remain poorly understood. Although detection of JCPyV has been demonstrated in the kidneys (39, 123), to our knowledge, there have been no studies demonstrating the infection of urine-derived archetype JCPyV and its replication and/or production of infectious virions in kidney cells. Furthermore, we previously demonstrated productive *in vitro* infection of primary human brain microvascular endothelial (HBMVE) cells by rearranged JCPyV (Mad-1A) (35). However, whether archetype JCPyV can infect and replicate in primary renal proximal tubule epithelial (RPTE) cells, HBMVE cells, and human brain cortical astrocytes (HBCA) has yet to be determined. In this study we conclusively demonstrate that archetype JCPyV can be propagated, that archetype JCPyV can infect and efficiently replicate in primary RPTE and HBMVE cells, while abortive replication occurs in HBCA cells and nonproductive infection occurs in primary human fetal glial (PHFG) cells, and rearrangement of urine-derived archetype JCPyV occurs *in vitro* in COS-7 cells at 645 days after infection.

Results

Isolation of archetype JCPyV from urine

Urine samples from 17 healthy volunteers were screened for the presence of JCPyV (Supplemental Fig. 1A) by qPCR of JCPyV TAg. Of the 17 volunteers, 13 had detectible JCPyV TAg genome copies, while four volunteers tested negative. JCPyV NCCR sequence analysis was conducted for samples that tested qPCR positive for JCPyV TAg. NCCR sequences of urine-derived JCPyV from five volunteers were compared with archetype and rearranged JCPyV sequences from Genbank (Supplemental Fig. 1B). In this study, we have screened, isolated, and confirmed via sequence analysis that a proportion of healthy volunteers excrete archetype JCPyV in their urine (Supplemental Fig. 1 and Supplemental Table 1).

Propagation of archetype JCPyV in COS-7 cells

COS-7 cells were infected with rearranged JCPyV to ensure their susceptibility to JCPyV infection. Semi-confluent COS-7 cells, grown in 35 mm plates, were inoculated with 0.5, 1 (data not shown), or 5 HA units of rearranged JCPyV and replication kinetics was measured by qPCR and qRT-PCR to determine the optimal infecting dose. COS-7 cells were then innoculated with archetype JCPyV isolated from urine to assess whether archetype JCPyV can be propagated using COS-7 cells as previously suggested (94). COS-7 cells were innoculated with 41 HA of urine-derived archetype JCPyV isolated from patient 7 (Supplemental Fig. 1A) and JCPyV replication was monitored by qPCR and qRT-PCR. JCPyV TAg and VP1 viral DNA and RNA transcripts were detected as early as 24 hr and 5 days after infection, respectively, increasing exponentially in parallel (Fig. 1A). The total JCPyV TAg (4.7×10^{11}) and VP1 (7.3×10^{11}) genome copies recovered from each 35 mm plate 25 days after infection were approximately

373- and 3.7 x 10^3-fold higher than the mean genome copies used to infect (infecting dose, ID), suggesting efficient replication of archetype JCPyV in COS-7 cells.

To further test the specificity of JCPyV TAg specific primers, we employed JCPyV and SV40 TAg specific primers and probes to amplify COS-7 cells infected with JCPyV. We demonstrate that JCPyV TAg specific primers and probe are specific to JCPyV with no cross reaction to SV40 TAg. As indicated in (Supplemental Fig. 2A), both control and JCPyV infected samples demonstrate a basal expression of SV40 TAg as compared to the house-keeping gene GAPDH in COS7 cells. This is in contrast to JCPyV (Supplemental Fig. 2B) where we see a steady increase of JCPyV TAg cDNA/mRNA over the course of 25 days when compared to GAPDH.

HA of combined cell lysate and supernatant collected at day 35 after infection confirmed the presence of archetype JCPyV virions in COS-7 cells. Due to the anticipation of a lower concentration of archetype JCPyV virions in infected COS-7 cells, a lower starting dilution for the HA was used, where we demonstrate approximately 21 HAU archetype JCPyV per µL. The replication kinetics and HA of rearranged JCPyV-infected COS-7 cells was also assessed (Fig. 1B). Due to the anticipation of a higher concentration of rearranged JCPyV virions in infected COS-7 cells, a higher dilution for the HA was used, where we demonstrate approximately 51 HAU of rearranged JCPyV per µL. These results demonstrate the potential to amplify infectious archetype JCPyV virions in COS-7 cells. Archetype JCPyV propagated and purified utilizing this method was used to conduct all pathogenesis studies described in this report using various susceptible cells.

Archetype JCPyV infection in primary HBMVE and RPTE cells

In at least three independent experiments, we examined the susceptibility of primary HBMVE and RPTE cells to archetype JCPyV infection and monitored the replication kinetics from days 1 to 20 after infection. Quantitative analysis of JCPyV TAg and VP1 genome copies and RNA transcripts was conducted by qPCR and qRT-PCR. Viral DNA was detected as early as 24 hr after infection, while RNA transcripts were detected as early as 5 days after infection in both HBMVE and RPTE cells. DNA and RNA transcripts increased exponentially after infection, where at day 20 the total JCPyV TAg (4.1×10^9) and VP1 (9.5×10^8) genome copies recovered from each 35 mm plate seeded with HBMVE cells were approximately 1.9×10^3- and 8.6×10^2-fold higher than the mean genome copies used for infection (Fig. 2A). In RPTE cells, the total JCPyV TAg (1.0×10^{12}) and VP1 (2.7×10^{10}) genome copies recovered were approximately 2.5×10^3- and 1.3×10^2 fold higher than the mean genome copies used for infection (Fig. 3A). Furthermore, JCPyV TAg protein was detected by IP/WB from archetype JCPyV-infected primary HBMVE (Fig. 2B) and RPTE (Fig. 3B) cells harvested 15 days after infection. At day 15, approximately 8% of primary HBMVE (Fig. 2C) and 6% of RPTE (Fig. 3C) cells expressed JCPyV VP1 protein using IFA. TEM confirmed the presence of viral particles with a diameter of 40 - 45 nm within the nucleus of HBMVE (Fig. 2D) and RPTE (Fig. 3D) infected cells. NCCR sequence analysis was conducted on archetype JCPyV infected HBMVE and RPTE cells, with no change in NCCR sequence when compared to DNA extracted from input archetype JCPyV used to infect and DNA extracted from any time point thereafter (Supplemental Fig. 2). To demonstrate the production of infectious archetype JCPyV virions, naïve HBVME and RPTE cells were infected with virus isolated from previously infected HBVME and RPTE cells collected at day 35 after infection (Fig. 4A and 4B). DNA replication and RNA transcripts in naïve cells were comparable to those observed in archetype JCPyV infected HBMVE and RPTE cells (Fig 1A and 2A).

Archetype JCPyV infection in primary HBCA and PHFG cells

Next we examined the susceptibility of primary HBCA to archetype JCPyV and compared its replication kinetics with that of rearranged JCPyV (Mad-1). JCPyV TAg and VP1 DNA copies and RNA transcripts (Fig. 5A) were detected in HBCA as early as 5 days after infection with archetype JCPyV, however, the genome copies and viral transcripts plateaued at day 10 after infection. At day 20 after archetype JCPyV infection, the total JCPyV TAg (3.1×10^8) and VP1 (3.6×10^7) genome copies recovered from each 35 mm plate of HBCA were approximately 27- and 33- fold higher than the mean genome copies used for infection. In contrast, rearranged JCPyV showed a steady increase of both DNA and RNA over the course of 20 days. At day 20 after Mad-1 JCPyV infection, the total JCPyV TAg (2.4×10^{10}) and VP1 (4.1×10^{10}) genome copies recovered from each 35 mm plate of HBCA were approximately 58- and 2.5×10^2- fold higher than the mean genome copies used for infection (Fig. 5B). Interestingly, neither JCPyV TAg protein was detected by IP/WB from archetype JCPyV-infected primary HBCA cells harvested 15 days after infection (Fig. 5C) nor VP1 staining was detected using IFA (data not shown). However, IFA demonstrated that approximately 1% of primary HBCA cells expressed JCPyV TAg protein at 15 days after infection (Fig. 5D).

On the basis that rearranged, but not archetype JCPyV infects PHFG cells (152), the susceptibility of PHFG cells to archetype JCPyV infection was examined. Archetype JCPyV TAg and VP1 genome copies were detected as early as day 1 after infection and declined slightly thereafter, while JCPyV TAg RNA transcripts were detected in PHFG cells as early as day 5 and declined steadily thereafter (Fig. 6A). VP1 RNA transcripts were not detected. Moreover, JCPyV TAg protein was not detected by IP/WB from archetype JCPyV-infected PHFG cells harvested at 15 days after infection (Fig. 6C). In contrast, rearranged (Mad-1) JCPyV replicated efficiently in PHFG cells. At day 20 after Mad-1 JCPyV infection, the total JCPyV TAg (2.2 x

10^{11}) and VP1 (5.3 x 10^{11}) genome copies recovered from each 35 mm plate of PHFG cells were approximately 1.7 x 10^3- and 5.0 x 10^3-fold higher than the mean genome copies used for infection (Fig. 6B).

In vitro rearrangement of urine-derived archetype JCPyV

NCCR sequence analysis was conducted on day 35 infected HBMVE, RPTE, COS-7, and re-infected naïve HBMVE and RPTE cells with JCPyV infected HBMVE and RPTE cell lysates, with no change in JCPyV NCCR sequences (Fig. 7). Interestingly, rearrangement of urine-derived archetype JCPyV NCCR occurred *in vitro* at 645 days after initial infection in COS-7 cells. This unique rearrangement resulted in one base pair substitution in block A, one base pair substitution in block B, an 8 base pair insertion in block C, and 5 base pair deletion in block F in the NCCR, with no changes to VP1. Sixteen binding sites present in CY, urine-derived archetype, and/or Mad-1 JCPyV were not present in D645 JCPyV as a result of its unique rearrangement (Table 1). As a result of the NCCR rearrangement of urine-derived archetype JCPyV altered replication kinetics were observed. Characterization of the replication kinetics of day 645 rearranged JCPyV (Fig. 8) demonstrated limited replication in HBMVE and RPTE cells, and non-productive replication in HBCA cells.

Discussion

Although we know that the archetype form of JCPyV circulates in the healthy human population (125) and that the rearranged form of JCPyV causes PML, the definitive route of transmission and the site(s) of primary replication prior to reactivation have not been clearly defined (84). It is thought that JCPyV infected lymphocytes and/or cell-free virus disseminates via the hematogenous route from primary sties of infection to secondary sites to establish focal areas of virus persistence (233). JCPyV has been detected in different tissues and organs in the human body including the tonsils (168), kidney (227, 261), bone marrow (227), brain (227), spleen (227), and gastrointestinal tract (192), however, it is unclear what specific cell type(s) and organs are permissive to archetype JCPyV infection, reactivation and rearrangement (114). The difficulty in delineating the cell types susceptible to archetype JCPyV infection has been a result of its restricted host cell range *in vitro* (70, 94, 114, 181). To address issues with the limited host cell tropism that JCPyV displays, studies have either focused on, but not limited to, using transformed cell lines to drive the replication of JCPyV and/or by introducing JCPyV DNA in cells via a plasmid based system (94). In this report, we demonstrate that urine-derived archetype JCPyV productively infects primary RPTE and HBMVE cells, while replication in HBCA cells is restricted and nonproductive, whereas the virus does not replicate in PHFG cells.

Propagation of urine-derived archetype JCPyV in COS-7 cells

One of the main difficulties in studying the natural history of archetype JCPyV acquisition, infection, and dissemination is the ability to isolate and/or propagate enough urine-derived archetype JCPyV to conduct these studies. To overcome the cumbersome task of isolating and propagating virus, investigators have employed a transfection-based system. An advantage to using such a system is having complete control of the JCPyV DNA used for transfection.

However, infection of urine-derived virus gives an opportunity to investigate naturally occurring variants independent of forced introduction of DNA where initial binding of virions and entry are ignored. In this regard, the progression of infection, including binding to the cell surface, entry, replication, and production of infectious virions can be studied in its entirety to understand archetype JCPyV cellular tropism. It has been reported that COS-7 cells support the replication of both archetype and rearranged JCPyV (94). In vivo data suggests that heterogeneous populations of PML-type NCCRs are ultimately derived (74, 104, 239) from the archetypal form of JCPyV over a period of time. Here we demonstrate that the period of time to propagate urine-derived archetype JCPyV *in vitro*, 35 days, is long enough for amplification of infectious virions with the archetype-like phenotype, but a period of time much shorter than that found *in vitro* to create variants. As demonstrated by other groups, the COS-7 cell is an effective *in vitro* model to propagate archetype JCPyV (94, 177). Utilizing this method, we were able to investigate the infection potential, replication kinetics, and cellular tropism of naturally occurring archetype JCPyV isolated from urine in different primary cells.

Archetype JCPyV infects and replicates in primary RPTE and HBMVE cells

JCPyV infection in human cell culture has been restricted to glial, astrocytic, neuroblastoma, Schwann, and B-cell lymphoma cells (114). Of these studies, only the rearranged form of JCPyV was used to demonstrate susceptibility to infection, while studies demonstrating archetype JCPyV susceptibility utilized a transfection-based approach and/or non-human derived cells. JCPyV variants with archetype NCCR have been detected in the urine of immunocompetent persons, as well as JCPyV DNA in kidneys of non-PML persons (261) suggesting that JCPyV establishes a low-level persistent infection in the kidneys of healthy persons. However, the exact cell type that archetype JCPyV infects within the kidney and its ability to replicate and produce infectious virions has not been established until now. A previous

study demonstrated the expression of JCPyV TAg protein in RPTE cells but did not demonstrate the presence of DNA, RNA, or JCPyV virions (152). Although transfection can address the contribution of intracellular components, like DNA-binding proteins, in JCPyV transcription and DNA replication, transfection bypasses the question of binding potential of JCPyV to host cell receptors involved in the entry of permissive cell types. It has been shown that both α-2,3- and α-2,6-sialic acid receptors are present on epithelial cells within the kidney (92, 258) and therefore should be susceptible to JCPyV binding and entry. Our data clearly demonstrate the ability of archetype JCPyV to productively infect, replicate, and produce infectious virions in RPTE cells.

Interestingly, there has been no data demonstrating the productive infection of archetype JCPyV in brain cells *in vitro* to support data found in vivo in which archetype JCPyV can be found in brains of healthy persons (228). Before delineating the mechanism of archetype JCPyV reactivation and rearrangement it is important to study the replication potential of archetype JCPyV in primary cell types of importance to PML pathogenesis. JCPyV latency in the brain prior to severe immunosuppression has remained inconclusive and controversial. Recent data demonstrates the presence of archetype JCPyV DNA in the brains of healthy controls (11, 228). Although rearranged Mad-1 JCPyV has been shown to productively infect and replicate in primary HBMVE cells (35), to our knowledge, this is the first study demonstrating that archetype JCPyV can infect and efficiently replicate in primary HBMVE cells. Our data, based on the expression of JCPyV early and late DNA, mRNA, protein, as well as the presence of archetype JCPyV virions and presence of infectious virions collectively demonstrate the productive infection and replication of archetype JCPyV in primary HBMVE cells. It is possible that prior to reactivation and rearrangement, archetype JCPyV is able to infect HBMVE cells that line the

blood-brain barrier (BBB) in immunocompetent persons, suggesting HBMVE cells susceptibility to infection after initial dissemination in the periphery.

In archetype JCPyV infected RPTE and HBMVE cells, the observation of higher late VP1 RNA transcripts could be a result of TAg protein mediated autoregulation similarly demonstrated in SV40 models in which late viral RNA was synthesized at a higher level than early RNA (198). It has been noted in SV40 models that an accumulation of TAg protein results in binding to the NCCR, thus repressing early transcription (106).

Restricted replication of archetype JCPyV in primary HBCA cells

In addition to the detection of JCPyV in the CSF or brain, histopathological identification of enlarged oligodendroglial nuclei, bizarre astrocytes, and demyelination can be used to further confirm diagnosis of PML. Although *in vitro* data demonstrate the susceptibility of astrocytes to rearranged JCPyV after transfection (69), astrocyte susceptibility to archetype JCPyV infection has not been demonstrated to date. It is known that the NCCR of rearranged JCPyV contains transcription factor binding sites due to its repeat structure (70) which is conducive to viral gene expression and its possible promiscuousness in infecting different primary cells and cell lines *in vitro*. Our findings demonstrate that primary HBCA cells infected with archetype JCPyV results in an abortive replication phenotype where the early TAg protein is produced without the VP1 protein or virion production. Similarly, the mechanism of the abortive replication in nonpermissive cells after treatment with monoclonal antibodies has been suggested to arise from the expression of the main viral oncogenic protein TAg in concert with other host tumor-inducing factors (13). The inability of archetype JCPyV to productively infect HBCA cells may be due to the structure of its NCCR and the lack of appropriate transcription factor binding sites

identified in rearranged JCPyV (157). In addition, the cellular transcription factor profile in HBCA cells might differ from cells that show productive infection of archetype JCPyV. However, the physiological constituents of the BBB make it feasible that astrocytes that surround and stabilize the capillary endothelial cells via their perivascular endfeet may be the next sequential cell to be infected after HBMVE cell infection due to its close proximity. It is therefore possible that archetype JCPyV can traverse the BBB before rearrangement and reactivation, and find residence in HBCA cells where non-productive replication may occur during primary infection. Data has suggested that viral propagation and amplification occurs in an astrocytic reservoir prior to oligodendrocytic infection in vivo (126). Thus, only upon immunosuppression can reactivation and rearrangement of JCPyV occur within HBCA cells resulting in efficient replication.

Archetype JCPyV does not replicate in PHFG cells

The replication profiles of archetype and rearranged JCPyV differ in PHFG cells, a heterogeneous population of glial cells. While rearranged JCPyV replicates efficiently in PHFG cells (32), archetype JCPyV fails to produce infectious virions (49). Consistent with previous findings, our data demonstrate that archetype JCPyV does not replicate in PHFG cells reiterating that archetype JCPyV is incapable of effective replication in glial cells and must rearrange its NCCR before being able to effectively replicate in glial cells (223). It is believed that the phenotype of the archetype JCPyV NCCR may be conducive to maintaining a persistent infection in non-glial cells, as we have demonstrated in RPTE and HBMVE cells, but once immunosuppression occurs changes to the NCCR could result in the ability of rearranged JCPyV to permissively infect oligodendrocytes (6).

Constant expression of TAg drives rearrangement of urine-derived archetype JCPyV in COS-7 cells

To our knowledge, this is the first description of *in vitro* rearrangement of archetype JCPyV. Since it has been demonstrated that SV40 TAg has a greater DNA binding activity to the JCPyV NCCR, as well as being more efficient in directing replication than that of JCPyV's own TAg (21, 40, 143), we utilized COS-7 cells constitutively expressing SV40 TAg to propagate archetype JCPyV. A previous study showed no rearrangements to the NCCR of JCPyV after transfection or infection in COS-7 cells cultured for weeks (94), and herein we show that propagation of virus stocks for 35 days also results in no rearrangements. This discrepancy was attributed to the fact that the period of time these JCPyV transfected or infected COS-7 cells were cultured was much shorter than the persistence of archetype JCPyV in human hosts in vivo. Thus, to overcome this shortcoming we decided to conduct an ongoing infection in COS-7 cells with no designated end point, which resulted in rearrangement to archetype JCPyV at 645 days after infection. It is known that the replication kinetics of JCPyV is a slow process even in susceptible cells where TAg is already present, however, it becomes clear that once an accumulation of TAg occurs, JCPyV viral replication proceeds (94, 151). Although the exact mechanism of NCCR rearrangement has yet to be described, it has been postulated that viral-replication-dependent recombination events might be responsible for the generation of deletions and/or duplication in the NCCR of archetype JCPyV (107).

Although bioinformatics tools like PROMO are powerful, limitations in identifying all the possible transcription factors that can bind to JCPyV NCCRs are dependent on data incorporated into the PROMO database from previous published data. Here in, we described predicted transcription factors that bind to our *in vitro* rearranged D645 JCPyV. Although differences in transcription factor binding sites were identified when comparing the NCCRs of D645, Mad-1,

urine-derived, and CY JCPyV, the exact role of these distinct host transcription factor binding site have yet to be described as being critical for the replication, reactivation, and rearrangement of archetype JCPyV in permissive cells. Thus far, of the predicted transcription factors produced by PROMO in our study, eight have been experimentally shown by different groups to play regulatory roles in JCPyV transcription, including NFI/CTF (170, 191), c-Jun (119), PURA (30, 37, 38, 129), C/EBP beta (200, 252), NF-1(137, 170), SpiB (156, 158), AP-1(204), and RelA (190, 205, 252, 255). Current studies in our laboratory are focused on utilizing these predicted transcription factor binding sites to demonstrate the importance of host transcription factors in the natural history of JCPyV infection and PML pathogenesis. Future *in vitro* studies to characterize these sixteen transcription factors will be done to conclusively demonstrate their importance in JCPyV transcription regulation.

Conclusions

Although debatable, independent groups have demonstrated the presence of archetype and/or rearranged JCPyV in the brains of immunocompetent persons and patients that suffer from neurological disorders other than PML (63, 73, 228, 236, 250). These data suggest that the presence of JCPyV within the brain may be independent of one's immune status and may occur during primary JCPyV infection. Collectively, our *in vitro* data suggest that RPTE, HBMVE, and HBCA cells may be sites in which archetype JCPyV may remain latent after primary infection. Utilizing our methods identified in this report, we are now able to generate an archetype JCPyV stock, which can be utilized for further studies focused on rearrangement, reactivation, and ultimately PML pathogenesis. It is known that JCPyV has a strict restricted cellular tropism, thus our finding that archetype JCPyV can productively infect and replicate in primary HBMVE and RPTE cells may provide clues into the cellular and molecular mechanisms of archetype JCPyV tropism. The nonproductive infection of archetype JCPyV in HBCA cells, when compared to

previous data demonstrating productive infection of rearranged JCPyV in HBCA cells, may help us to understand the importance of the cellular environment for infection based on the JCPyV NCCR structure, which will allow development of therapeutics for PML. Our results support the idea that JCPyV may have a propensity for maintaining a persistent infection in non-glial cells (6). Archetype JCPyV may lay latent in peripheral organs such as the kidneys, in HBMVE cells that line the BBB, or HBCA cells within the brain of asymptomatic persons, where immunosuppression can lead to reactivation and rearrangement into the neurotropic form. Although we demonstrated *in vitro* rearrangement of archetype JCPyV, the exact mechanism of rearrangement is unknown. Thus studies are currently underway to delineate the possible molecular mechanisms of rearrangement and reactivation of archetype JCPyV. Our overarching goal is to elucidate the transcription factor profile of cells permissive to JCPyV infection in hopes of understanding the cellular environment conducive to reactivation and rearrangement.

Materials and Methods

Cell culture

COS-7 cells were maintained as described previously (94). Primary HBMVE cells and primary HBCA cells were purchased from Cell Systems Corporation and maintained as previously described (35, 240), while primary RPTE cells (Cat #4100) were purchased from Sciencell. HBCA, HBMVE, and RPTE cells between passages P6 and P8 were used in all experiments. Fetal brain tissues were obtained from the Kapiolani Medical Center for Women and Children (KMCWC), after receiving approval from the KMCWC Institutional Review Board and the University of Hawai'i Committee on Human Studies (UHCHS) and processed as described previously to generate PHFG cells (32).

Virus

Archetype JCPyV was isolated from the urine of healthy volunteers after obtaining written consent and study approval by UH-CHS. Urine was received from patients, stored at 4^{o}C no longer than 12 hr, and processed as previously described (71). Urine samples were not pooled. DNA was extracted using Qiagen QIAprep Spin Miniprep Kit according to the manufacturer's protocol from 100 µL of processed sample. Urine-isolated JCPyV was then quantitated by real-time PCR (qPCR) (32) and confirmed by sequencing NCCR as described previously (202). To generate archetype virus stock, COS-7 cells were infected with urine-derived JCPyV and harvested at day 35 after infection. Virus isolation and purification was conducted as previously described (32). Virus was then quantitated by HA assay (234) and qPCR, and confirmed by NCRR sequence analysis.

HA assay

VP1 is the major capsid protein of the JCPyV and it is responsible for the attachment to cells and agglutinates human type O erythrocytes (234). Human type O erythrocytes were centrifuged at 2,500 rpm for 10 min at 4^{o}C, washed twice in Alsever's buffer (20 mM sodium citrate, 72 mM NaCL, 100 mM glucose, pH 6.5 adjusted with acetic acid), and suspended in Alsever's buffer at a final concentration of 0.5%. Serial two-fold dilutions of virus suspensions were prepared in Alsever's buffer. 50 µL of viral suspension and an equal volume of RBC were added to each well of a 96-well "U" bottom microtiter plate and incubated at 4^{o}C for 3-6 hr, with a final volume of 100 µL. The final dilution of virus suspension that agglutinates red blood cells was considered the end point of the titration and read as the reciprocal of that dilution. The end point dilution is considered 1 hemagglutination (HA) unit, with the estimated ratio of infectious particles being approximately 10^4 to 1 HA unit (33, 173).

JCPyV infection

1x10^5 COS-7, RPTE, HBMVE, HBCA, and PHFG cells were seeded in tissue culture treated 35 mm plates to study viral kinetics, and 1x10^6 cells in T-75 tissue culture flasks were seeded for protein extraction. Additionally, 5x10^4 cells were seeded in each well of a 24-well plate containing cover slips for immunofluorescence assay (IFA). At 80-90% confluency, aforementioned cells were either mock-infected with medium only, or inoculated with 41 HA JCPyV per 1x10^5 cells. Initial virus inoculums were measured using qPCR, prepared at appropriate concentrations, and added into designated plates, wells, or flasks and returned to an incubator (37^{o}C with 5% CO_2) for 24-hr adsorption for archetype JCPyV and 2-hr adsorption for rearranged JCPyV. Each plate, well, or flask was then washed twice with 1X PBS to remove unadsorbed virus followed by replenishment of fresh medium. Wells, plates, and flasks were

kept at 37°C with 5% CO_2 until time of cell harvest at designated time points. Culture medium was changed every 2 days.

Serial passaging of COS-7 cells for 665 days

At 80-90% confluency, cells were infected with 41 HA per $1x10^5$ cells of archetype JCPyV per T-75 tissue culture flask. Infected cells were passaged every 10 days, and cell lysates were collected for DNA and RNA analysis.

Reinfection of naïve cells

At 80-90% confluency, cells were infected with 41 HA per $1x10^5$ cells of archetype JCPyV per T-75 tissue culture flask. Infected cells were passaged every 10 days, and cell lysates were collected for DNA and RNA analysis. For the reinfection of naïve HBMVE or RPTE cells,

thirty five days after infection, infected cells were subjected to virus isolation and purification as previously described (32). Supernatant/initial virus inoculum was measured using qPCR and naïve HBMVE or RPTE cells were reinfected as mentioned above.

DNA and RNA extraction and quantitative analysis

Low molecular weight DNA and total RNA were extracted from mock- and archetype JCPyV-infected cells from 35 mm plates harvested on days 1 (24 hr after infection), 5, 10, 15, 20, and 25 after infection as previously described (32). cDNA was synthesized from 1 µg of cellular RNA using Bio-Rad iScript cDNA synthesis kit following the instructions provided by the manufacturer. JCPyV DNA or cDNA was amplified using 2 µL of template DNA or cDNA, 10

pmol each of forward and reverse primers, and probe specific for JCPyV TAg and for VP1 genes in a final reaction volume of 20 µL as previously published by our group (32). qPCR was conducted using a Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Detection System. Analysis was conducted via Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Optical System Software Version 3.1.

PCR amplification and sequence analysis

JCPyV NCCR was amplified using 2 µL of template DNA and primers JRR-25 and JRR-28 as described previously (202). PCR products were separated on a 2% agarose gel, visualized with ultraviolet light, and purified by QIAquick PCR purification column and sequenced for positive identification of archetype JCPyV. Utilizing PROMO, a web-based program which utilizes the TRANSFAC database of transcription factor binding motifs, potential transcription factor binding sites were predicted for JCPyV sequenced NCCRs (163).

Immunoprecipitation and western immunoblot

Total cellular protein was extracted from mock-infected and JCPyV-infected cells and separated by centrifuging the lysate for 30 min at 12,000 rpm at 4^{o}C. Protein concentrations were assayed using Bio-Rad Quick Start Bradford Protein Assay. T antigen from 250 µg of total protein extracts were immunoprecipitated using 60 µL of protein G Plus/Protein A Agarose suspension and 10 µL (2 µg) of anti-SV40 T antigen mouse mAb at 4^{o}C overnight (35). Immunoprecipitated protein was separated on SDS-PAGE, transferred onto nitrocellulose membranes, and incubated overnight using anti-SV40 T antigen mouse primary antibody (1:1,000) as described previously (35). Following incubation with secondary antibodies conjugated with IRDye 680

(1:10,000) (Li-Cor Biosciences), the membranes were visualized using the Li-Cor Odyssey imaging system according to manufacturer's instructions (199).

Immunofluorescence assay

HBMVE, RPTE, and HBCA cells were seeded on coverslips in 24-well pates ($5x10^4$ cells/well). Cells were either mock-infected with medium only, or infected with JCPyV. Cell preparation and staining with various primary antibodies were conducted as previously described (35). Fluorescent cells were examined using an Axiocam MRm camera mounted on a Zeiss Axiovert 200 microscope equipped with the appropriate fluorescent filters and objectives.

Transmission electron microscopy

After 15 days, mock-infected and JCPyV-infected HBMVE and RPTE cells cultured in 35 mm plates were washed twice with cold 1 X PBS, treated with serum-free trypsin-EDTA solution and spun at 13,000 rpm for 10 mins at 4^{o}C. The supernatant was decanted and the cell pellet was fixed with 2.5% glutaraldehyde in 0.1M sodium cocodylate buffer, pH 7.4, for 1-2 hr at room temp. Fixed samples were processed using a Hitachi HT7700 all-digital 120 kV Transmission Electron Microscope (TEM) with AMT 2k x 2k CCD camera and tomography option for conventional TEM.

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Figure Legends

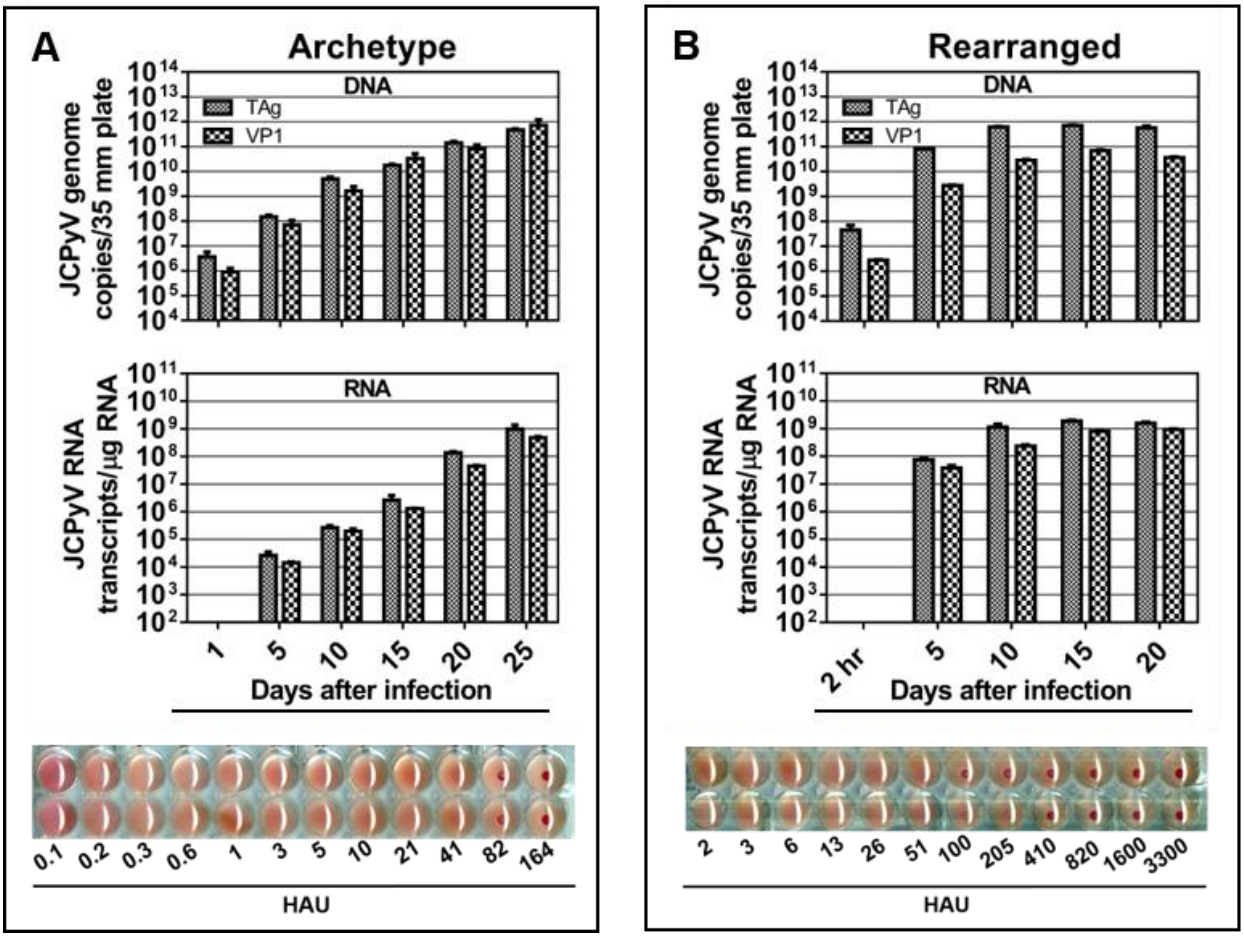


Figure 1. Urine-isolated archetype JCPyV efficiently replicates in COS-7 cells: Semi-confluent COS-7 cells were infected with either (A) 41 HA urine-isolated archetype or (B) 5 HA Mad-1 JCPyV and cells were harvested at indicated time points for DNA and RNA extraction. Viral TAg and VP1 genome copies, and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively. HA assay was conducted using human 'O' blood group positive RBC, to confirm the production of archetype and rearranged type JCPyV virions. HAU, hemagglutination assay units.

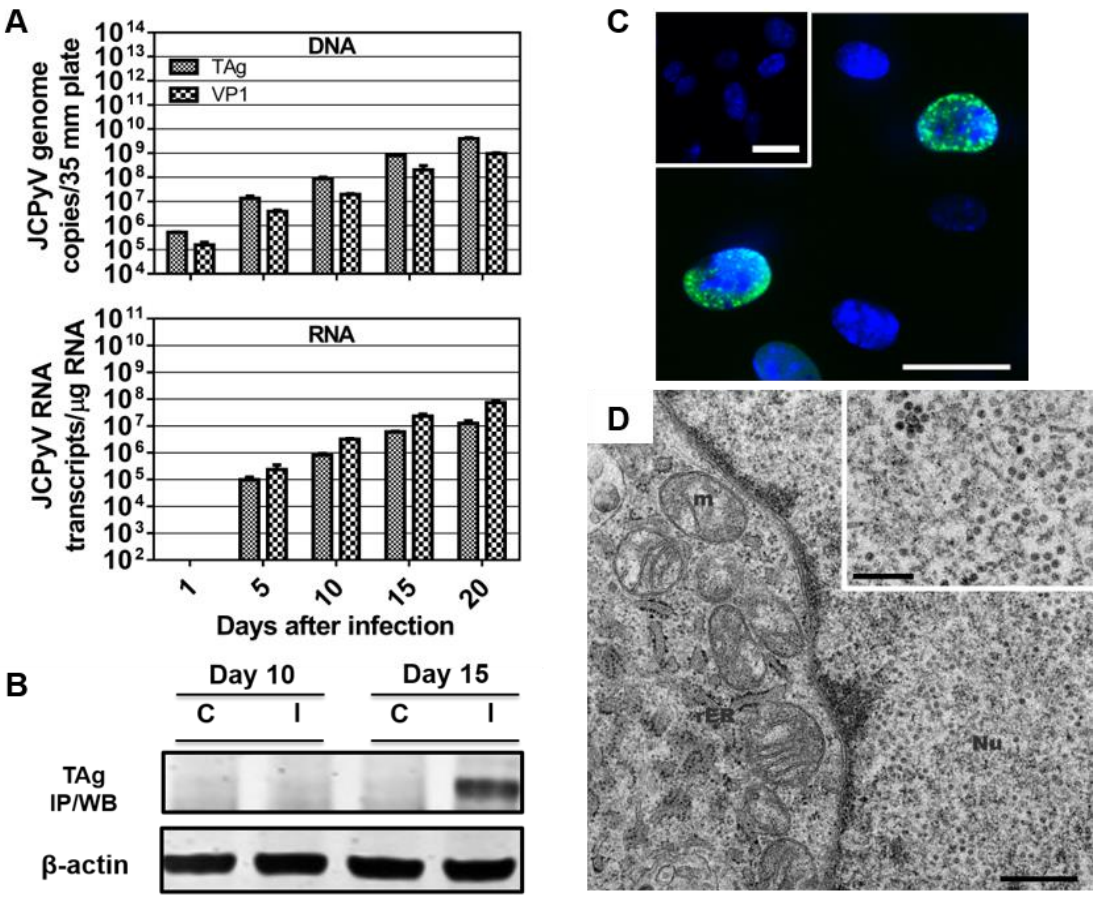


Figure 2. Archetype JCPyV efficiently replicates in primary HBMVE cells: HBMVE cells were infected with 41 HA of COS-7 cells propagated urine-isolated archetype JCPyV for 24 hr and cells were harvested at indicated time points. DNA and RNA were extracted and viral TAg and VP1 (A) genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively. (B) IP followed by WB analysis was conducted on JCPyV infected HBMVE cell lysates harvested at day 15 after infection using anti-SV40 TAg mouse mAb. C, control uninfected; I, infected. (C) IFA was conducted on HBMVE cells infected with archetype JCPyV at day 15 after infection and cells were stained using anti-JCPyV VP1 mouse mAb; inset indicates secondary Ab only; VP1 (green) and DAPI (blue); scale bar, 20 µm. (D) TEM was conducted for detection of JCPyV virions in the nucleus of primary HBMVE cells. Scale bar, 500 nm; inset scale bar, 250 nm. M, mitochondria; Nu, nucleus; rER, rough endoplasmic reticulum.

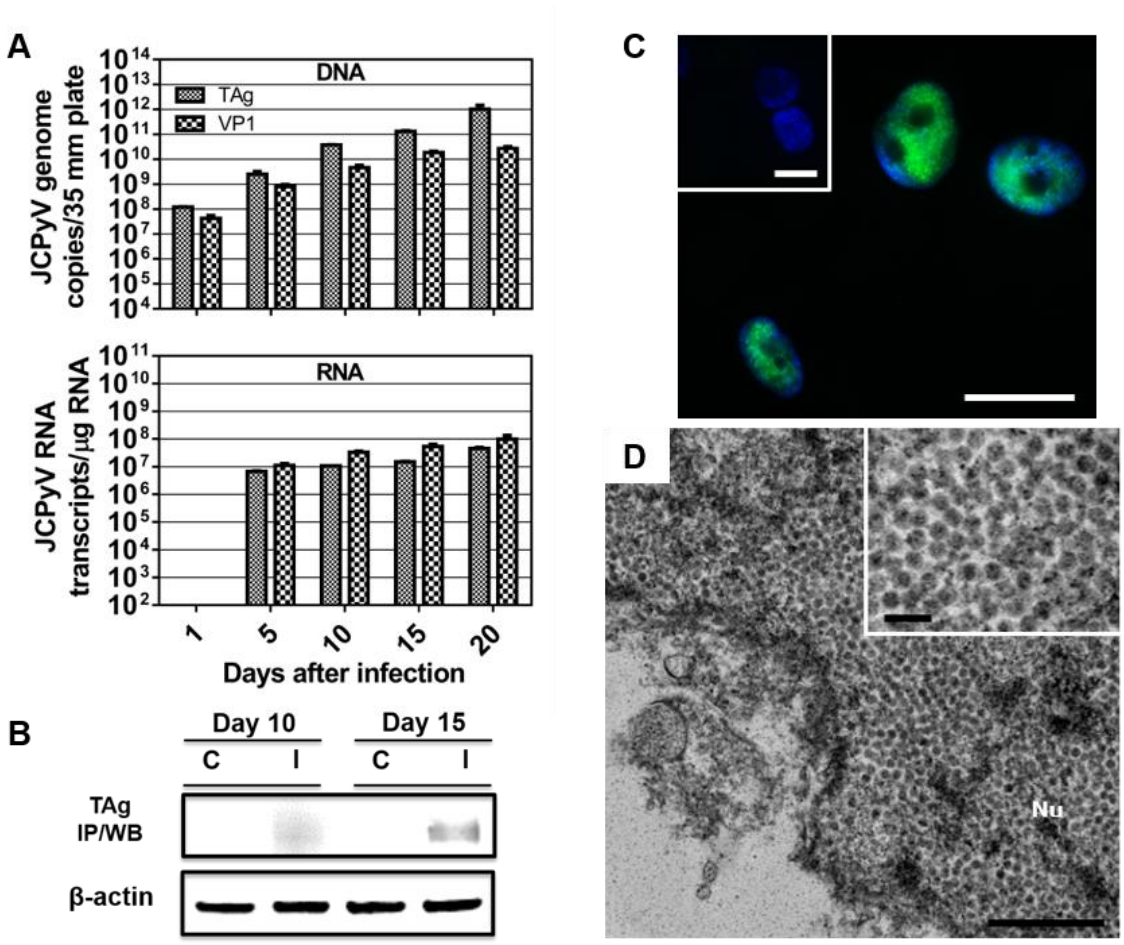


Figure 3. Archetype JCPyV efficiently replicates in primary RPTE cells: RPTE cells were infected with 41 HA of COS-7 cells propagated urine-isolated archetype JCPyV for 24 hr and cells were harvested at indicated time points. DNA and RNA were extracted and viral TAg and VP1 (A) genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively. (B) IP followed by WB analysis was conducted on JCPyV infected HBMVE cell lysates harvested at day 15 after infection using anti-SV40 TAg mouse mAb. C, control uninfected; I, infected. (C) Immunofluorescence staining was conducted on HBMVE cells infected with archetype JCPyV at day 15 after infection and cells were stained using anti-VP1 mouse mAb; inset indicates secondary Ab only; VP1 (green) and DAPI (blue); scale bar, 20 µm. (D) TEM was conducted for detection of JCPyV virions in the nucleus of primary RPTE cells. Scale bar, 500 nm; inset scale bar, 250 nm. Nu, nucleus.

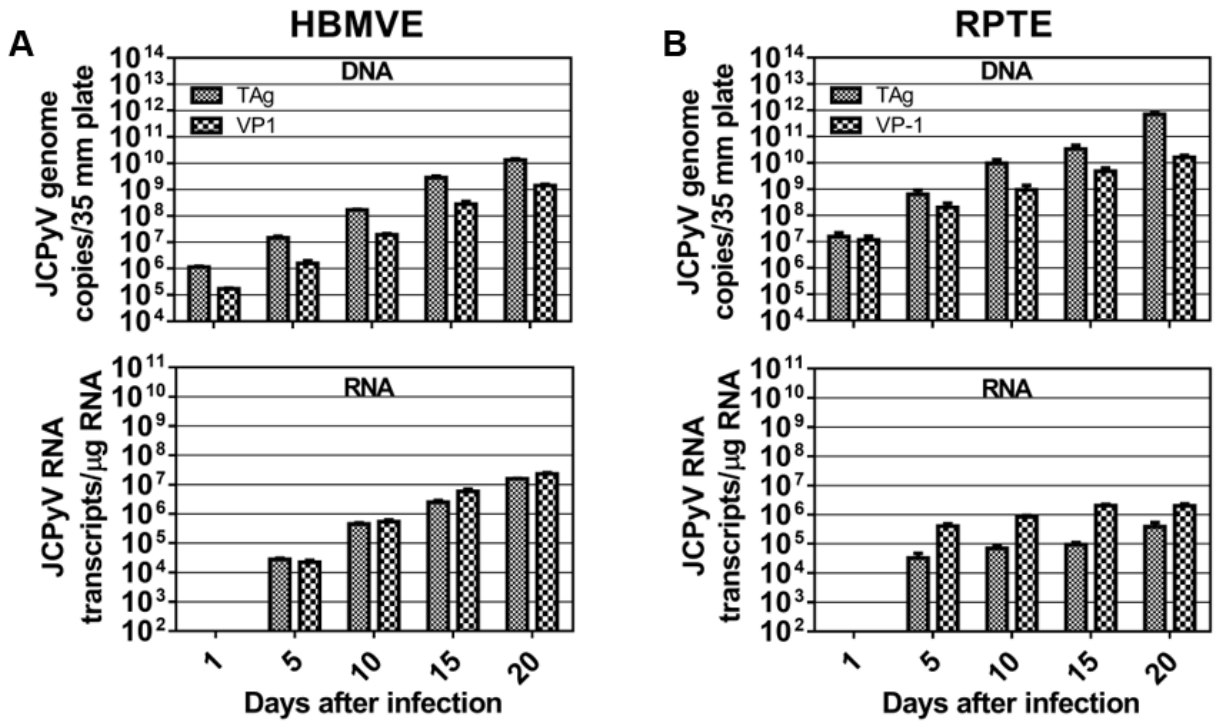


Figure 4. Reinfection of naïve HBMVE and RPTE cells demonstrates production of infectious virions: Naïve HBMVE and RPTE cells were infected with 41 HA of archetype JCPyV isolated from previously infected HBMVE and RPTE cells, and cells were harvested at indicated time points for DNA and RNA extractions. Reinfection of isolated archetype JCPyV in HBMVE cells TAg and VP1 (A) genome copies and RNA transcripts. Reinfection of isolated archetype JCPyV in RPTE cells TAg and VP1 (B) genome copies and RNA transcripts.

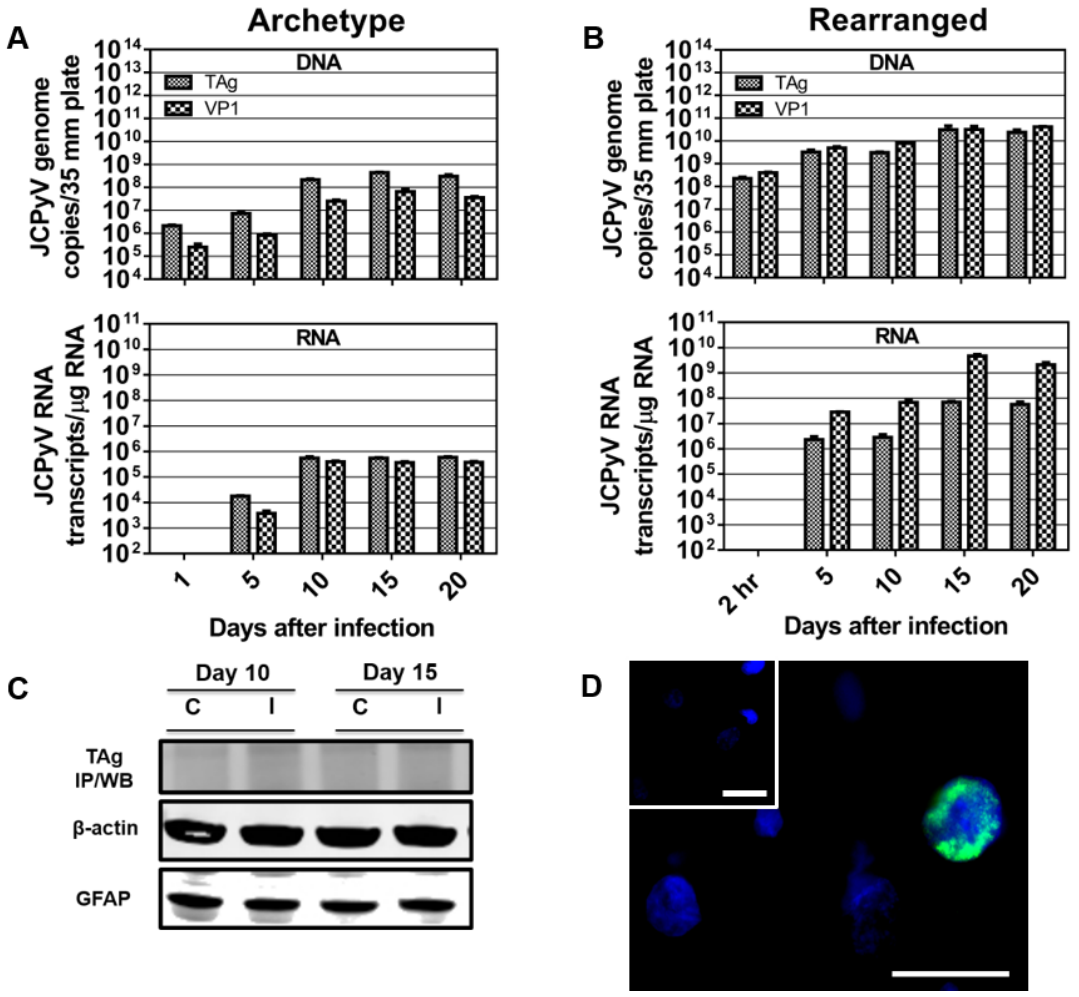


Figure 5. Limited replication of archetype JCPyV in primary HBCA: HBCA were infected with 41 HA of either COS-7 cells propagated urine-isolated archetype JCPyV or Mad-1 JCPyV, and cells were harvested for DNA and RNA extractions on indicated days. Archetype JCPyV TAg and VP1 (A) genome copies and RNA transcripts or Mad-1 JCPyV (B) TAg and VP1 genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively. (C) IP followed by WB analysis was conducted on JCPyV-infected HBCA cell lysates harvested at day 15 after infection using anti-SV40 TAg mouse mAb. C, control uninfected; I, infected. (D) IFA was conducted on HBCA infected with archetype JCPyV at day 15 after infection and cells were stained using anti-SV40 TAg mouse mAb; inset indicates secondary Ab only TAg (green) and nucleus (DAPI, blue); scale bar, 20 µm.

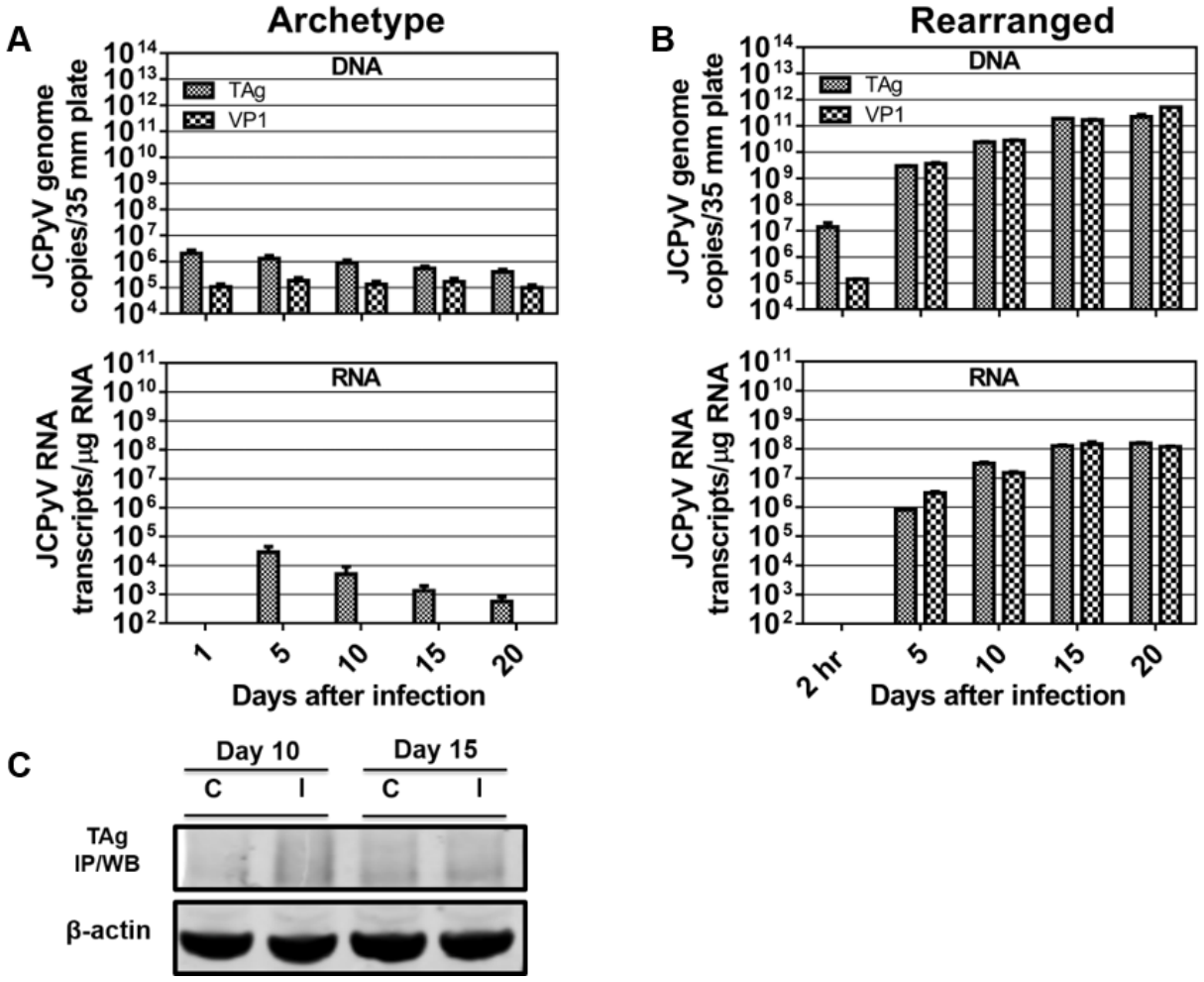


Figure 6. Archetype JCPyV does not replicate in PHFG cells: PHFG cells were infected with 41 HA of either COS-7 cells propagated urine-isolated archetype JCPyV or Mad-1 JCPyV, and cells were harvested at indicated time points for DNA and RNA extractions. Archetype JCPyV TAg and VP1 (A) genome copies and RNA transcripts or Mad-1 JCPyV TAg and VP1 (B) genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively. (C) IP followed by WB analysis was conducted on JCPyV-infected PHFG cell lysates harvested at day 15 after infection using anti-SV40 TAg mouse mAb. C, control uninfected; I, infected.

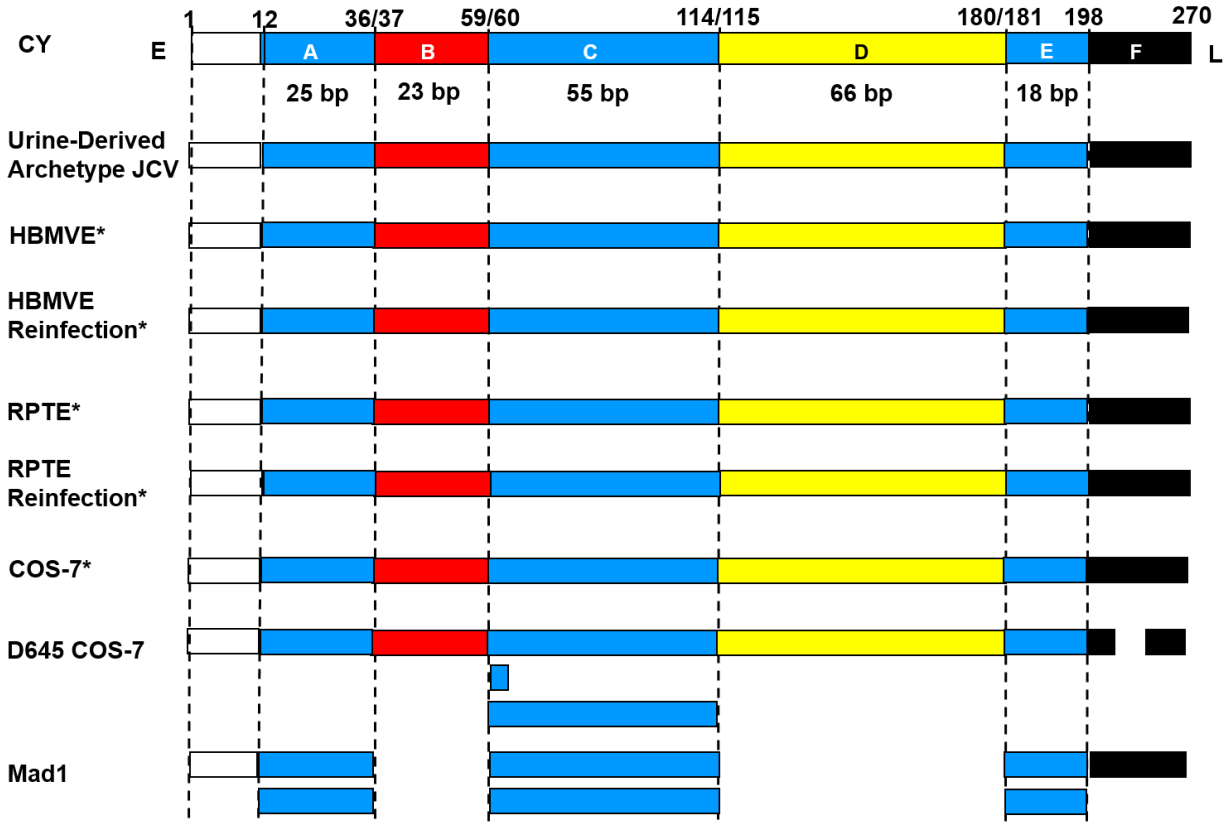


Figure 7. NCCR comparison of archetype JCPyV infected primary cells demonstrate conservation in the NCCR but rearrangement in COS-7 cells at 645 days Conventional PCR was conducted for JCPyV NCCR with primers JRR-25 and 28. The nucleotide start position at 1 in the CY, archetype JCPyV [M35834], is that of Yogo et al. [1990]. Mad1, rearranged JCPyV [J02227], contains duplicate copies in block A, a 25-bp region containing the TATA box, block C, a 55-bp region, and block E, an 18-bp region, to yield a 98-bp tandem repeat. Sequences encoding the early proteins (E), large T, small t, and T', are to the left of the nucleotide start position 1. The initial codon for the agnoprotein is at position 270. Sequences encoding the late proteins (L), VP1, VP2, and VP3 are to the right of block F. *Archetype JCPyV NCCR sequences at 35 days after infection with respective cell types. D645 COS-7, urine-derived archetype JCPyV passaged every 10 days demonstrated rearrangement at 645 days after infection, contains one base pair substitution in block A, one base pair substitution in block B, an 8 base pair insertion in block C, and 5 base pair deletion in block F.

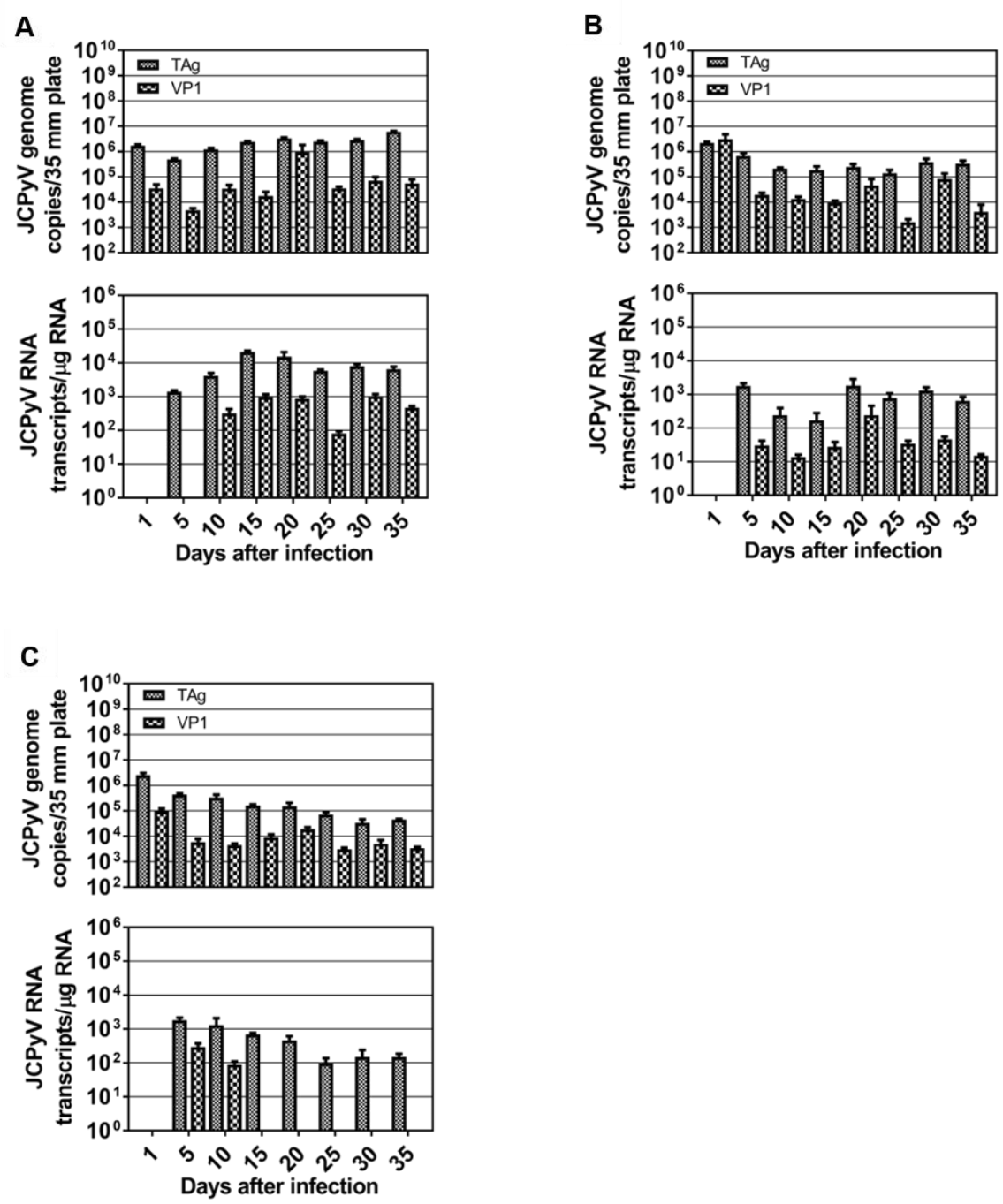


Figure 8. Replication kinetics of day 645 rearranged JCPyV infected primary cells (A) HBMVE, (B) RPTE, and (C) HBCA cells were infected with 41 HA of D645 rearranged JCPyV, and cells were harvested for DNA and RNA extractions on indicated days. JCPyV TAg and VP1 genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR, respectively.

Table 1. Predicted transcription factor binding sites

Transcription Factor [Transfac ID][a]		JCPyV D645[b]	JCPyV Mad-1[c]	JCPyV Urine[d]	JCPyV CY[e]	Experimental Evidence[f]
GR-beta [T01920]		+	+	+	+	
C/EBP alpha [T00105]		+	+	+	+	
C/EBP beta [T00581]		+	+	+	+	+
AP-4 [T00036]		+	+	+	+	
AP-3 (2) [T00039]			+	+	+	
NF-1 [T00539]		+	+	+	+	+
TGIF [T04076]		+	+	+	+	
RAR-gamma [T00720]		+	+	+	+	
Pax-2 [T01823]		+	+	+	+	
Myf-5 [T00521]		+	+	+	+	
Tal-1 [T00790]		+	+	+	+	
AIRE [T05990]		+	+	+	+	
c-Fos [T00123]		+	+	+	+	
PKNOX1 [T04122]		+	+	+	+	
AP-2alphaA [T00035]		+	+	+	+	
Pax-6 [T01122]		+	+	+	+	
TFIIB [T00818]		+	+	+	+	
Hlf [T01071]		+	+	+	+	
GCMa [T02306]		+		+	+	
Nrf2 [T01443]		+	+	+	+	
NF-AT2 [T01945]		+	+	+	+	
NF-AT1 [T01948]		+	+	+	+	
HMGI(Y) [T02368]		+	+	+	+	
STAT4 [T01577]		+	+	+	+	
c-Ets-1 [T00112]		+	+	+	+	+ (SpiB)
STAT1beta [T01573]		+	+	+	+	
HNF-1B [T01950]		+		+	+	
HNF-1C [T02918]				+	+	
VDR [T00885]		+		+	+	
TFIID [T00820]		+	+	+	+	
EBF [T05427]		+		+	+	
BTEB3 [T05051]		+	+	+	+	
R1 [T00711]		+		+	+	
AP-1 [T00029]		+				+
FOXN2 [T04206]		+		+	+	
Pu box binding factor [T00704]		+				
MBF1 [T00492]			+	+	+	
Cart-1 [T03978]				+	+	
ENKTF-1 [T00255]			+	+	+	
RelA [T00594]				+	+	+
DBP [T04875]				+	+	
AR [T00040]			+	+	+	
FOXP3 [T04280]				+	+	
TGIF [T04076]				+	+	
HSF1 (long) [T01042]				+		
HSF1 (short) [T02104]				+		
AREB6 [T00625]			+	+	+	

Transcription Factor [Transfac ID][a]		JCPyV D645[b]	JCPyV Mad-1[c]	JCPyV Urine[d]	JCPyV CY[e]	Experimental Evidence[f]
P300 [T01427]		+	+	+	+	
TMF [T00835]		+	+	+	+	
CDX2 [T03246]			+	+	+	
STAT5A [T04683]		+	+	+	+	
Elk-1 [T00250]		+	+	+	+	
Nkx2-1 [T00857]		+	+	+	+	
E47 [T00255]		+	+	+	+	
c-Myb [T00137]		+	+	+	+	
YY1 [T00915]		+	+	+	+	
PEA3 [T00685		+	+	+	+	
LF-A1 [T00467]		+	+	+	+	
NFI/CTF [T00094]		+	+	+	+	+
LCR-F1 [T01599]		+	+	+	+	
Pbx1 [T06000]		+	+	+	+	
Myf-3 [T00519]		+	+	+	+	
MyoD [T00525]		+	+	+	+	
USF2b [T02377]		+	+	+	+	
USF2 [T00878]		+	+	+	+	
c-Jun [T00133]		+	+	+	+	+
NF-E2 [T00558]		+				
NHP-1 [T00621]		+	+	+	+	
NF-X3 [T01514]		+	+	+	+	
p53 [T00671]		+		+	+	
EllaE-A [T00246]		+	+	+	+	
POU2F2C [T00665]		+		+	+	
POU2F2 (Oct-2.1) [T00646]				+	+	
POU3F2 [T00630]				+	+	
TBP [T00794]		+	+	+	+	
R2 [T00712]		+	+	+	+	
MAZ [T00490]		+	+	+	+	+ (PURA)
FOXO3a [T02938]		+	+	+	+	
SRY [T00997]		+	+	+	+	
FOXJ2 (long isoform) [T04169]		+	+	+	+	
HNF-3beta [T02513]		+	+	+	+	
TCF-1A [T00999]		+	+	+	+	
LEF-1 [T02905]		+	+	+	+	
TCF-4E [T02878]		+	+	+	+	
TCF-4 [T02918]			+	+	+	
MZF-1 [T00529]		+	+	+	+	
GABP-alpha [T01390]		+	+	+	+	
GABP [T00268]		+	+	+	+	
IRF-1 [T00423]		+	+	+	+	
IRF-3 [T04673]		+	+	+	+	
NF-AT1 [T00550]		+	+	+	+	
PR B [T00696]		+	+	+	+	
PR A [T01661]		+	+	+	+	
GR-alpha [T00337]		+	+	+	+	

[a] Common name and Transfac ID of putative binding transcription factors

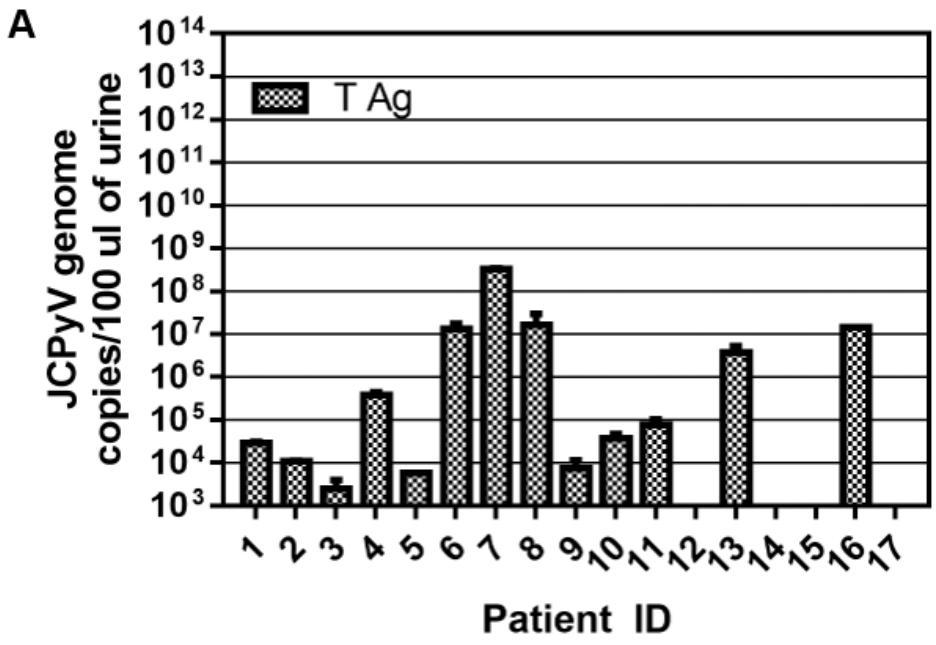
[b] Transcription factors that bind the promoter of D645 COS-7 rearranged JCPyV

[c] Transcription factors that bind the promoter of Mad-1 variant of JCPyV

[d] Transcription factors that bind the promoter urine-derived archetype JCPyV

[e] Transcription factors that bind the promoter of CY JCPyV

[f] Experimental evidence demonstrating binding of transcription factors to JCPyV NCCR



B

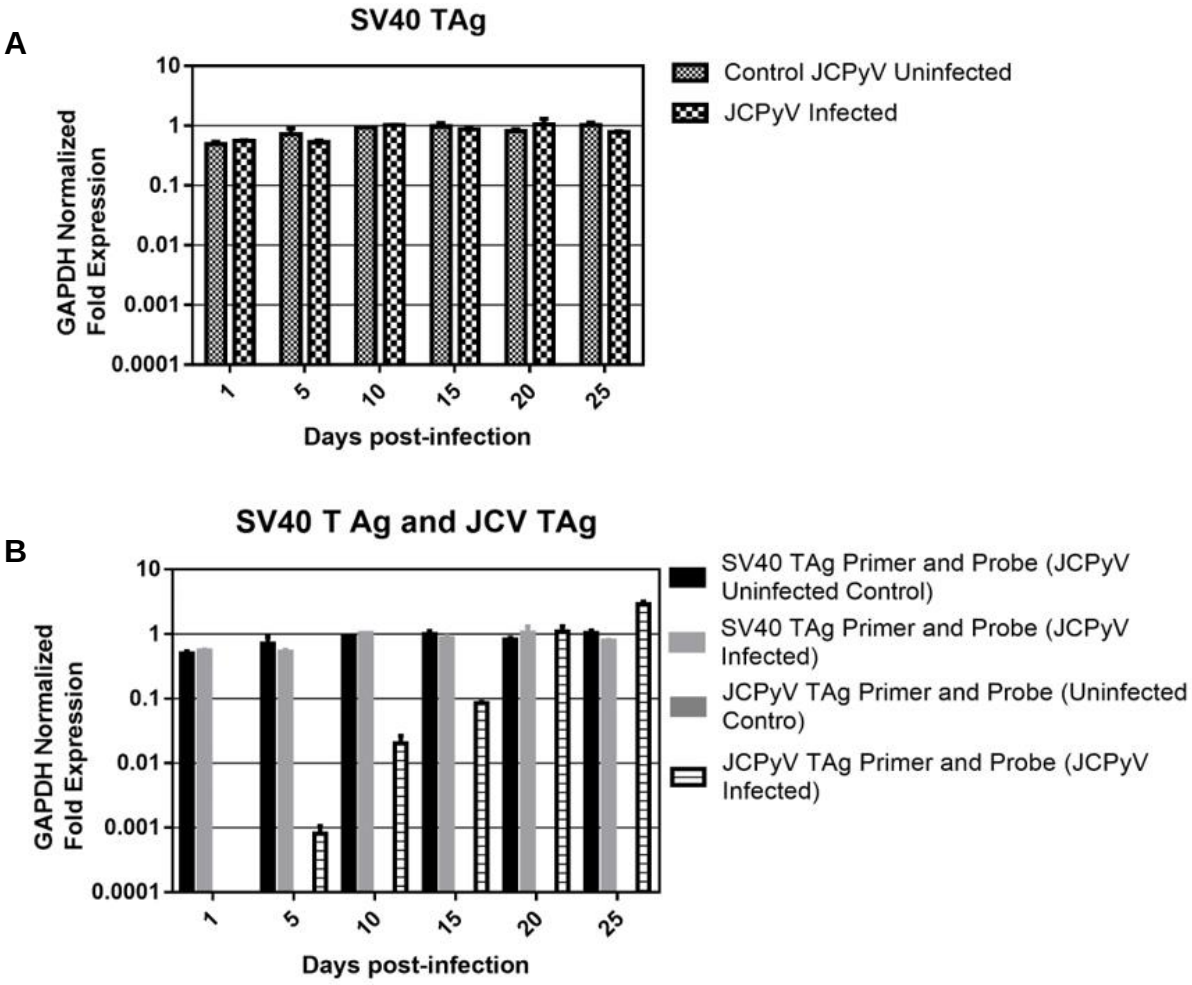
Percent Identity

	Mad-1	CY	6	7	8	13	16	
Mad-1		77.4	77.9	79.2	78.6	76.7	76.8	Mad-1
CY	15.9		99.6	100.0	99.6	99.2	99.6	CY
6	16.4	0.4		99.6	99.2	97.7	99.3	6
7	15.9	0.0	0.4		99.6	97.7	99.6	7
8	16.6	0.4	0.8	0.4		98.5	99.2	8
13	16.3	0.4	0.8	0.4	0.4		98.9	13
16	16.5	0.4	0.8	0.4	0.8	0.8		16
	Mad-1	CY	6	7	8	13	16	

Divergence

Patient ID

Supplemental Figure 1. Isolation of archetype JCPyV from urine: (A) Early morning urine samples from healthy volunteers were screened for the presence of JCPyV TAg genome and were quantitated using qPCR. (B) Amplification of the NCCR by PCR with JCPyV specific NCCR primers JRR-25 and 28 and sequence analysis by ClustalW demonstrate percent identity and divergence of JCPyV positive patient IDs compared to CY [M35834] and Mad1 JCPyV [J02227].



Supplemental Figure 2. JCPyV real-time primers and probes are specific to JCPyV: (A) qRT-PCR of JCPyV infected COS-7 cells using SV40 TAg specific primers and probe. (B) Comparison of SV40 TAg specific primers and probe and JCPyV TAg specific primers and probe.

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Author Contributions

N.L. and V.R.N. designed, analyzed results, and wrote the manuscript. N.L. conducted the experiments. N.L. and V.R.N. analyzed data. All authors have read and approved the final version of the manuscript.

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CHAPTER 4

EFFECT OF ARCHETYPE JCPYV VP1 MUTATIONS ON REPLICATION KINETICS IN PRIMARY BRIAN CELLS AND ITS CONTRIBUTIONS IN MECHANISMS OF JCPYV PATHOGENESIS

Effect of archetype JCPyV VP1 mutations on replication kinetics in primary brain cells and its contributions in mechanisms of JCPyV pathogenesis

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Abstract

Background: Progressive multifocal leukoencephalopathy (PML), caused by the polyomavirus JC (JCPyV), remains an important cause of morbidity and mortality among AIDS patients. While archetype JCPyV is widespread and circulates in humans, only the rearranged type of JCPyV causes PML. We have previously demonstrated that urine-derived archetype JCPyV replicates in human brain microvascular endothelial (HBMVE) cells. However, the role of the viral capsid protein 1 (VP1) coding region in PML pathogenesis is still unclear. Based on our preliminary data, we hypothesized that mutations in VP1 will result in JCPyV increased infection and replication in HBMVE cells. **Methods:** Primary HBMVE cells were transfected with 25 ng of either parental constructs of archetype JCPyV (CY), rearranged JCPyV (Mad1 or M1), or hybrid JCPyV (CYrM1c), which contains an archetype NCCR in the backbone of rearranged JCPyV (Mad1) coding region. HBMVE cells were also transfected with 25 ng of either CYrM1c constructs with the VP1 mutations CYrM1c-S267F or CYrM1c-L55F. Cells were harvested at 4 hr and at 3, 5, 10 and 15 days after transfection, and DNA and RNA was extracted to study virus replication kinetics using qPCR and qRT-PCR for JCPyV early and late genes, TAg and VP1 respectively. In addition, primary human fetal glial (PHFG) cells were transfected with 25 ng M1, M1-L55F, or M1-S267F, amplified via VP1 PCR, and sequenced. Lysate from PHFG cells transfected with M1, M1-L55F, or M1-S267F were sonicated, tittered, and used to reinfect PHFG cells to demonstrate production of infectious virions. Lastly, COS-7 cells were transfected with 25 ng of CY to propagate infectious virus used to infect HBMVE cells. VP1 and NCCR sequence analysis was conducted for each experiment. **Results:** Input JCPyV TAg and VP1 DNA was detected for all three JCPyV constructs 4 hrs after transfection. Archetype JCPyV (CY) and rearranged JCPyV (Mad1) replication increased steadily over the course of 15 days after transfection, whereas increase in replication was not observed using the hybrid JCPyV (CYrM1c). **Conclusions:** These preliminary data demonstrate that archetype JCPyV (CY) and

rearranged JCPyV (Mad1) can replicate in HBMVE cells, whereas limited replication was observed when HBMVE cells were transfected with the hybrid JCPyV (CYrM1c). Studies are ongoing to understand transcription of viral RNA via qRT-PCR, protein production by immunoprecipitation followed by western blotting for JCPyV TAg, and NCCR rearrangements via sequence analysis.

Introduction

Despite the ubiquitous nature of JCPyV, with reports depicting seroprevalence ranging between 66% to 92% (242) and up to 40% of the population persistently shedding virus in the urine (124), progressive multifocal leukoencephalopathy (PML) is rare and almost always associated with an underlying immunosuppressive condition. PML is a fatal demyelinating disease caused by the reactivation of latent JCPyV resulting in the lytic infection of oligodendrocytes, the myelin producing cells within the CNS. Although JCPyV is generally asymptomatic, factors leading to immunosuppression or immune dysfunction, such as the use of immunosuppressive drugs or HIV/AIDS, can trigger the reactivation, replication, and lytic infection of JCPyV in oligodendrocytes resulting in PML. Currently, there are no treatments for PML and is fatal within a few months from onset. The only proven approach to manage PML in affected individuals is the reversion of the immune suppression when possible (42, 149). Although JCPyV reactivation in individuals with a compromised immune system is associated with PML, the exact mechanisms leading to PML remains unknown.

It is thought that active replication results in the accumulation of deletions and duplications within the noncoding control region (NCCR) (70, 138, 187) and point mutations in the viral capsid protein 1 (VP1 (113, 263) of archetype strains, giving rise to neurotropic rearranged stains. Neurotropic rearrangements are independent amongst affected individuals, but these rearrangements always occur in the NCCR and often also in VP1 (212).

It is believed that both host (i.e. transcription factors) and viral genetics, NCCR rearrangement and VP1 mutations, may contribute to the development of PML. Several studies have reported the presence of mutations in the major VP1 in JCPyV isolated from PML patients (113, 225, 262, 263). These nonpolymorphic (i.e. JCPyV subtype-independent) PML-associated mutations

or deletions of JCPyV VP1 include the amino acid positions 50, 51, 55, 60, 61, 122-125, 265, 267, 269, 271, and 283 (113, 262, 263). Cinque et al reported that the most frequent VP1 changes involved the amino acids 269 and 55, with VP1 mutants 55F, 267F, and 269F having a loss in hemagglutination property (87). These studies suggest the importance of VP1 in JCPyV pathogenesis in the context of VP1 mediated immune responses (127, 246), cell attachment, and viral entry via sialic acid receptors (36, 135). Although viral isolates with VP1 mutations have been demonstrated to be present in individuals with PML, it has not been established whether JCPyV with PML-associated VP1 mutations are pathogenic in cells of the CNS.

We have previously demonstrated that rearranged Mad-1 (35) and archetype JCPyV (unpublished data) productively infects primary human brain microvascular endothelial (HBMVE) cells, cells that line the blood-brain barrier (BBB). Therefore, the objective of this study was to assess the relationship between VP1 mutations associated with PML (i.e., mutants L55F, S267F, and S269F) and the JCPyV replication kinetics in HBMVE cells.

Results and Discussion

JCPyV VP1 mutants are replication-incompetent in primary HBMVE cells

To define the replication kinetics of PML-associated JCPyV VP1 mutations in HBMVE cells, we transfected primary HBMVE cells with five different JCPyV constructs. These constructs included three parental strains and two PML-associated VP1 mutants: 1) CY, consisting of the archetype NCCR and coding regions; 2) Mad-1, consisting of the rearranged NCCR and coding regions; 3) CYrM1c, consisting of the archetype NCCR and Mad-1 coding regions; and 4) L55F; and 5) S267F mutants, consisting of the archetype NCCR and Mad-1 coding regions with the amino substitutions in VP1 at positions 55, leucine to phenylalanine, and 267, serine to phenylalanine. As expected, Mad-1 transfection of HBMVE cells resulted in an increase in DNA and mRNA transcripts of both TAg and VP1 over the course of 35 days (Fig. 1D). Our previously published data also demonstrated productive infection of Mad-1 JCPyV in HBMVE cells (35). Interestingly, CY transfection of HBMVE cells resulted in a decrease of TAg and VP1 DNA over 35 days, with the detection of TAg and VP1 mRNA at only day 5 after transfection (Fig. 1A). CYrM1c (Fig. 1B), L55F (Fig. 1D), and S267F (Fig. 1E) displayed a similar trend, where a gradual decrease in TAg and VP1 DNA was observed over 35 days with detection of TAg and VP1 mRNA only at day 5 after transfection.

Glucocorticoid treatment of HBMVE cells fails to enhance JCPyV transcription

Dexamethasone, a synthetic glucocorticoid, has the ability to suppress interferon responses resulting in strong immunosuppressive properties (75, 142, 206). *In vivo* experiments have demonstrated increased virus replication in mammalian cells after dexamethasone treatment for Brennan-Krohn polyomavirus (BKPyV) (165), herpes simplex virus 1 (HSV-1) (57, 96), murine mammary tumor virus (MMTV) (108, 184), and the retrovirus Moloney murine leukemia virus

(MMLV) (222). In rare instances, case reports of JCPyV induced PML in the context of organ transplantation has been described as an adverse side effect of glucocorticoid use. In 24 cases of PML associated with transplant recipients reported in the literature, nine occurred in renal transplant patients, six in bone marrow, 4 in liver, three in heart, and two in lung transplants. All of the solid-transplant recipients received immunosuppressive treatment, with eighty-three percent of the patients receiving corticosteroids (216). In this study, we demonstrate that dexamethasone treatment is not toxic to HBMVE cells (Table 1). The physiological concentration of dexamethasone used was based off of previous reports, where 50 ng/mL of dexamethasone resulted in an 11-fold increase in BKPyV viral capsid protein expression (165). In contrast to BKPyV, HSV-1, MMTV, and MMLV, dexamethasone failed to enhance TAg and VP1 transcription of CY, CYrM1c, and Mad-1 JCPyV strains (Fig. 2). The increase in virus replication and transcription rate, particularly the late genes expressing structural proteins, of BKPyV was attributed to the presence of a functional nonconsensus GRE/PRE sequence and a consensus ERE sequence located in the late leader (overlapping the putative agnongene start codon) of the BKPyV NCCR (165). It is plausible that the reason dexamethasone treatment of JCPyV infected HBMVE cells failed to show enhancement in viral replication and/or transcription could be due to the differences in the NCCR of JCPyV and BKPyV, where GRE/PRE and ERE sequences have been described in BKPyV but not in JCPyV.

Differences in infection and transfection of CY JCPyV in HBMVE cells

Cell signaling is strongly activated during viral infection and might facilitate viral uptake and appropriate intracellular signaling following binding of viruses to receptors (89). Therefore, discrepancies between infection and transfection in JCPyV might be plausible. Interaction between the virus and host cell receptor in susceptible cells results in activation and triggering of a signaling cascade, which primes a favorable cellular environment for completing the virus

life cycle (23). For example, in T-cells, within a minute of HIV-1 infection, more than 200 phosphorylation sites are modified, potentially altering several cellular processes resulting in an environment conducive to virus replication (53). We previously reported that archetype JCPyV can productively infect HBMVE cells (unpublished), therefore it was surprising to observe that CY transfection in HBMVE cells failed to replicate (Fig. 1A). Utilizing similar methods to propagate urine-derived archetype JCPyV, we were able to propagate infectious CY virions (Fig. 3A) to corroborate that CY, like urine-derived archetype JCPyV, can infect HBMVE cells. Although delayed, CY virions are capable of replicating as TAg and VP1 DNA and mRNA were detected by qPCR and qRT-PCR (Fig. 3B). The artificial insertion of exogenous viral DNA into cells via transfection is an important, well-established tool. However, one must appreciate the natural history of viral infection and the importance of bypassing the initial step of infection. As mentioned, binding to the permissive host cell receptor elicits downstream pathways and molecules that are important for virus replication. Transfection is a complex process that can produce both direct (intended) and indirect (unintended) results, thus having the potential to cause biological responses that are unrelated to what is being transfected (109).

Propagation of infectious virions in PHFG cells fails to reinfect naïve HBMVE cells

To further evaluate the difference between infection and transfection in the context of JCPyV, we utilized similar methods in virus propagation for Mad-1 VP1 mutants as previously described (35). Herein, virus from previously transfected PHFG cells was used to reinfect naïve HBMVE cells. DNA copies from previously transfected PHFG cells was used to calculate the TAg and VP1 DNA (2×10^8 average) used to reinfect naïve HBMVE cells (Fig. 4A). Mad-1 S267F and Mad-1 strains, but not Mad-1 L55F, had sufficient amount of virus to reinfect naïve HBMVE cells for 35 days. As expected, Mad-1 reinfection of naïve HBMVE cells resulted in productive infection, showing an increase in both TAg and VP1 DNA and mRNA. In contrast, the Mad-1 S267F mutant failed to replicate over the course of 35 days.

Conclusion

In vivo data demonstrates JCPyV L55F and S267F VP1 mutations as being the most common mutations in CSF-derived sequences. This observation was correlated with the ability of both mutations to abrogate JCPyV hemagglutination and binding to peripheral cells and to sialic acid, suggesting this loss of function as being advantageous (87). It has been speculated that changes in glycan specificity would allow JCPyV to lose its specificity to sialylated glycans expressed outside of the CNS (e.g. red blood cells). Interestingly, it has been demonstrated that HBMVE cells express α2,3 and α2,6 -linked sialic acid receptors (1). Thus, it is reasonable to believe that JCPyV VP1 mutants L55F, S267F, and S267F would not be able to bind and infect HBMVE cells. We were unable to demonstrate this possibility due to the fact that transfection of JCPyV VP1 mutants L55F, S267F, and S267F failed to replicate and produce infectious virions. We were, however, able to demonstrate the propagation of CY virions after transfection in COS-7 cells and the ability of these virions to infect HBMVE cells.

Materials and Methods

Cell culture

COS-7 cells were maintained as described previously (94). Primary HBMVE cells were purchased from Cell Systems Corporation and maintained as previously described (35, 240). HBMVE cells between passages P6 and P8 were used in all experiments.

Plasmids

CY, CYrM1c, Mad-1, CYrM1c-L55F, and CYrM1c-S267F plasmids were a received as a gift from Dr. Richard Frisque (Pennsylvania State University). NCCR and VP1 plasmids sequences were confirmed as described previously (202). To generate CY virus stock, COS-7 cells were transfected with CY plasmid and harvested at day 35 after transfection. Virus isolation and purification was conducted as described previously, (32) followed by quantitation by qPCR.

DNA transfection

$1x10^5$ HBMVE cells were seeded on 35 mm 6-well plates to study viral kinetics. At 80-90% confluency, cells were either mock-transfected with medium only, or transfected with 25 ng of either CY, CYrM1c, Mad-1, CYrM1c-L55F, and CYrM1c-S267F plasmid using Lipofectamine® LTX & PLUS™ Reagent (Invitrogen), following the manufacturer's protocol. Six-well plates were kept at 37^{o}C with 5% CO_2 until time of cell harvest at designated time points. Culture medium was changed every 2 days.

Dexamethasone treatment

HBMVE cells were pretreated 24 h with dexamethasone prior to transfection. This was followed by continuous treatment until designated harvest time points. Culture media with dexamethasone was changed every third day. Dexamethasone was added to the culture media just before changing the media.

Reinfection of naïve cells

Approximately 10^5 HBMVE or PHFG cells were seeded in each well of the 6-well plates to conduct viral kinetics study. At 80-90% confluency, cells were either mock-treated with medium only, or infected with 41 HA per $1x10^5$ cells, where 3 mL of medium was used per 35-mm plate. Before infection medium from each well or flask was removed leaving medium just enough to cover the culture surface in each well/flask. Virus inoculums prepared at appropriate concentrations in 100 µL volume were then added into designated wells/flasks. Culture plates or flasks were then returned to a 37ºC incubator with 5% CO_2 for 24 hr adsorption for archetype JCPyV, while 2 hr adsorption for rearranged JCPyV. Each well/flask was then washed twice with 1X PBS to remove unadsorbed virus followed by replenishment of fresh medium. Plates and flasks were then kept at 37ºC with 5% CO_2 until time of cell harvest at specific time points. Cell medium was changed every 2 days.

DNA and RNA extraction and quantitative analysis

Low molecular weight DNA and total RNA were extracted from mock- and archetype JCPyV-infected cells grown in 35 mm plates harvested on days 1 (24 hr after infection), 5, 10, 15, 20, and 25 after infection as described previously (32). cDNA was synthesized from 1 µg of cellular

RNA using Bio-Rad iScript cDNA synthesis kit following the instructions provided by the manufacturer. JCPyV DNA or cDNA was amplified using 2 µL of template DNA or cDNA, 10 pmol each of forward and reverse primers, and probe specific for JCPyV TAg and for VP1 genes in a final reaction volume of 20 µL as previously published by our group (32). qPCR was conducted using a Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Detection System. Analysis was conducted via Bio-Rad iCycler iQ™ Multicolor Real-Time PCR Optical System Software Version 3.1.

PCR amplification and sequence analysis

JCPyV NCCR was amplified using 2 µL of template DNA and primers JRR-25 and JRR-28 as described previously (202). PCR products were separated on a 2% agarose gel, visualized with ultraviolet light, and purified by QIAquick PCR purification column and sequenced for positive identification of archetype JCPyV.

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sequence polymorphisms in the outer loops of the JC polyomavirus major capsid protein (VP1) possibly associated with progressive multifocal leukoencephalopathy. The Journal of general virology **86:**2035-2045.

Figure Legends

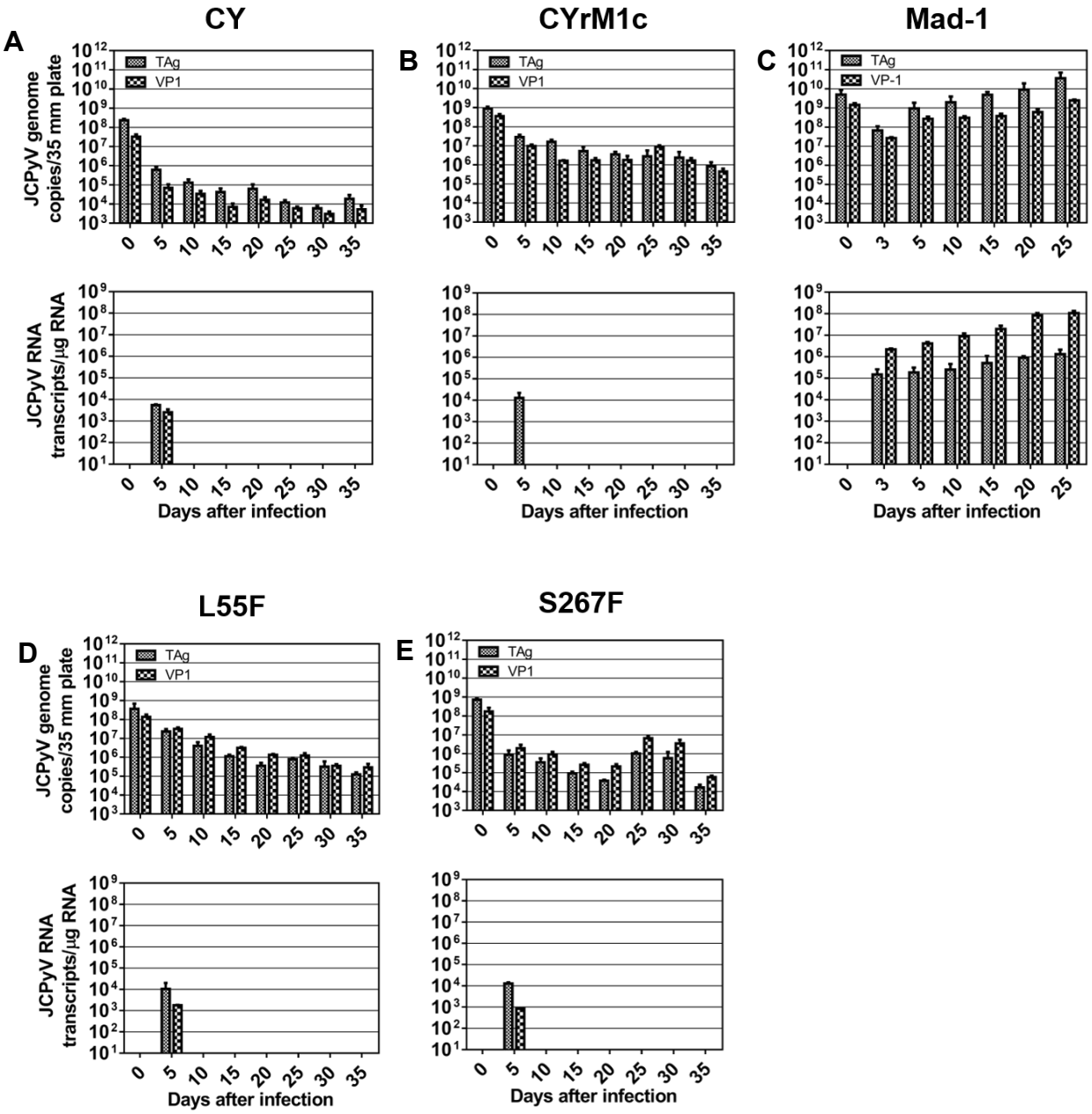

Figure 1. CY, CYrM1c, and CYrM1c VP1 mutants are replication-incompetent in primary HBMVE cells: HBMVE cells were transfected with 25 ng of either (A) CY, (B) CYrM1c, (C) Mad-1, (D) CYrM1c-L55F, or (E) CYrM1c-S267F JCPyV plasmids. Cells were harvested for low molecular-weight DNA and total cellular RNA extraction on indicated days. Viral TAg and VP1 genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR.

Table 1. Dexamethasone toxicity in HBMVE cells using CellTiter96® AQueous One Solution Cell Proliferation Assay

	Dexamethasone Concentration (ng/mL)			
	25	50	100	125
Days after treatment	*** Cell Survival % of Control (+/- SD)**			
1	107 (10)	102 (3)	100 (1)	100 (2)
3	100 (7)	97 (4)	99 (7)	98 (8)
5	93 (7)	102 (8)	98 (8)	93 (7)
10	88 (5)	89 (3)	85 (4)	84 (6)

*Data obtained from six wells of a 96 well plate
HBMVE: Human Brain Microvascular Endothelial cells

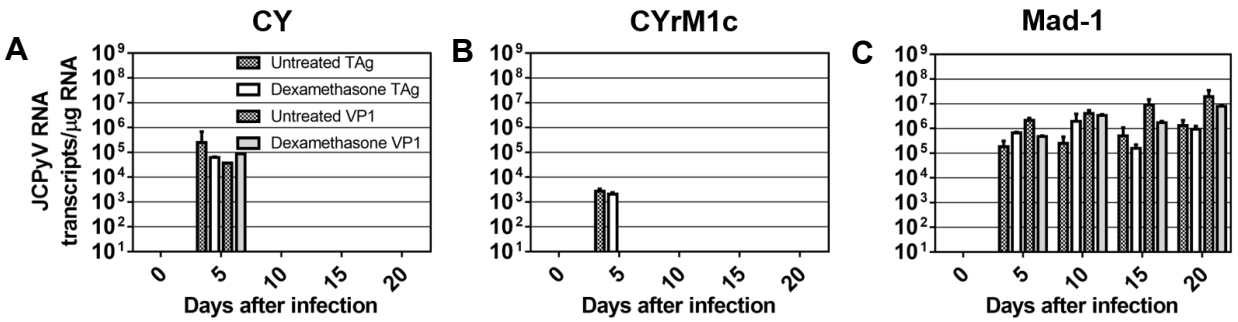

Figure 2. Effect of dexamethasone treatment on JCPyV RNA expression: HBMVE cells were either treated with 100 ng of dexamethasone for 24 h or treated with PBS before being transfected with (A) CY, (B) CYrM1c, or (C) Mad-1. In a 96 well plate, 10,000 HBMVE cells were plated per well. One day before transfection, cells were pre-treated with 100, ng/mL dexamethasone. Twenty-four hours after seeding, transfection reagents were added to each well following Invitrogen's Lipofectamine® LTX & PLUS™ Reagent protocol. At the designated time points days 1, 3, 5 and 10 the percent viable cells, compared to untreated control, were assayed using Promega's CellTiter 96® Aqueous ONE Solution Cell Proliferation Assay System following the manufacture's protocol. The absorbance at 490 nm was recorded using a 96-well plate reader.

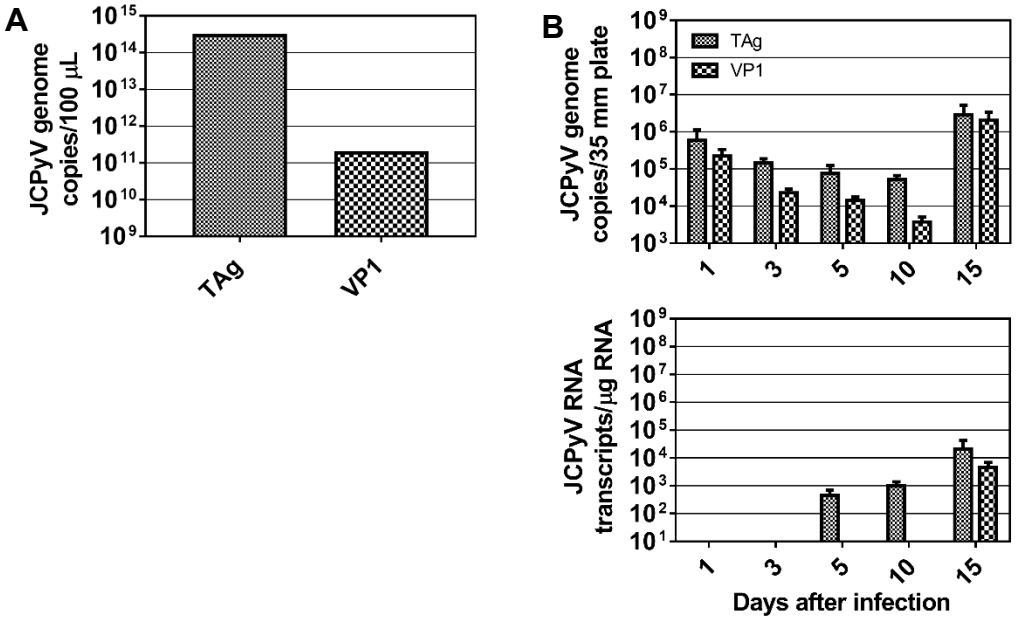

Figure 3. CY transfected COS-7 isolated virus infects HBMVE cells: Sucrose cushion isolated virus was prepared from 35-day COS-7 cells transfected with CY JCPyV. (A) COS-7 propagated CY JCPyV genome titers were determined by TAg and VP1 qPCR. (B) 41 HA of CY isolated virus from COS-7 cells were used to infect HBMVE cells. Viral TAg and VP1 DNA genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR

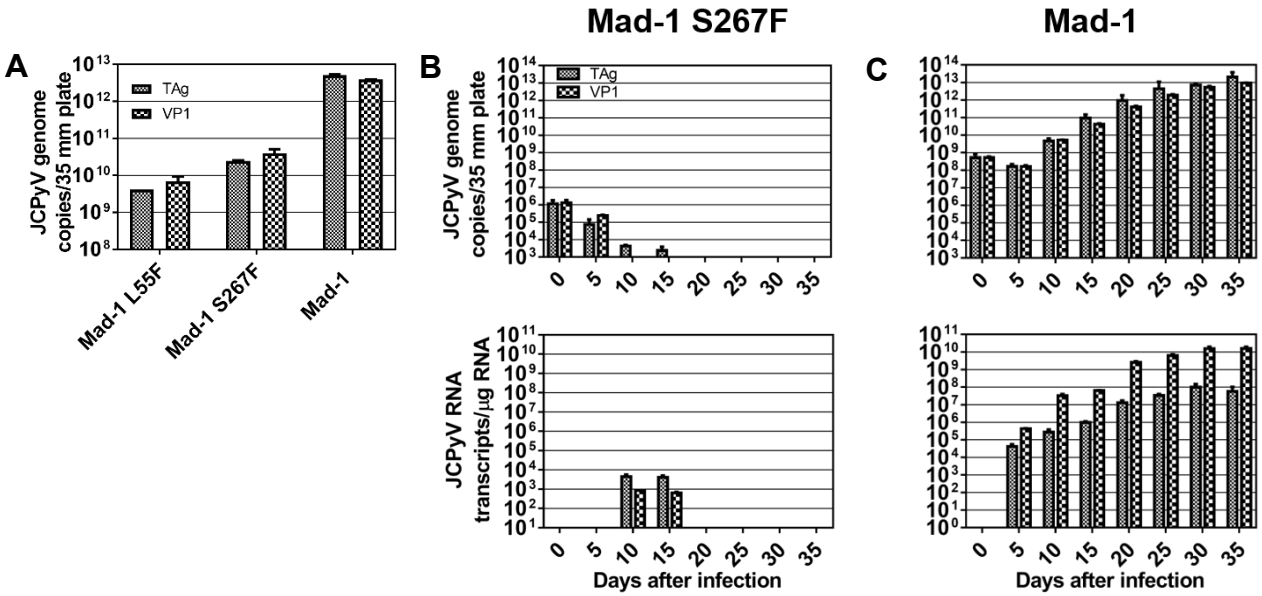

Figure 4. PHFG transfected Mad-1 VP1 mutant isolated virus are replication-incompetent after infecting HBMVE cells: PHFG isolated virus was prepared from 35-day PHFG cells transfected with Mad-1 L55F, Mad-1 S267F, or Mad-1 JCPyV. (A) Mad-1 L55F, Mad-1 S267F, or Mad-1 JCPyV genome titers were determined by TAg and VP1 qPCR. (B) 41 HA of Mad-1 S267F or Mad-1 isolated virus from COS-7 cells were used to infect HBMVE cells. Viral TAg and VP1 DNA genome copies and RNA transcripts were quantitated by qPCR and qRT-PCR

CHAPTER 5

PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY: DEVELOPMENT OF A HUMAN POLYOMAVIRUS JC INFECTION MODEL USING HUMANIZED MICE

Progressive multifocal leukoencephalopathy: Development of a human polyomavirus JC infection model using humanized mice

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Abstract

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were innoculated with archetype and rearranged human polyomavirus JCPyV and the course of ensuing infection was evaluated in a quest to develop and characterize an animal model to study the pathogenesis of JCPyV infection. Prior to inoculation, human immune cells engrafted in NSG mice were confirmed by flow cytometry analysis. NSG mice were inoculated with either archetype JCPyV or rearranged JCPyV by intravenous (IV) injection. Blood and urine were collected at days 3, 5, 7, 14, 21, and 28 after inoculation and JCPyV viral DNA and TAg protein in NSG mice was detected by real-time PCR (qPCR) and flow cytometry, respectively. Our data demonstrate that NSG mice have >50% CD45+ human cells as determined by the percentage of human CD45^{+} cells in the peripheral blood by flow cytometry. Both archetype JCPyV and rearranged JCPyV productively infected NSG mice. JCPyV TAg DNA was detected as early as day three after inoculation in urine of mice, which peaked at day 7 after inoculation. JCPyV TAg DNA was first detected in the blood of NSG mice on day seven after inoculation. JCPyV TAg protein was also detected in the blood on day 7 after inoculation as measured by flow cytometry. JCPyV TAg DNA was detected in the urine and blood of NSG mice up to two weeks after inoculation. This study demonstrates JCPyV can infect humanized NSG mice. Future research is focused on the routes of primary infection and mechanisms of reactivation after HIV co-infection.

Introduction

The human polyomavirus JC (JCPyV) is the etiological agent of progressive multifocal leukoencephalopathy (PML), a rare demyelinating disease of the brain caused by the viral lytic infection of oligodendrocytes in immunocompromised individuals. PML remains an important cause of morbidity and mortality among immunocompromised patients including patients with HIV/AIDS, malignancies, transplant recipients, and individuals treated with immunomodulatory drugs.

Studies have reported up to 80% of the healthy human population as being seropositive for JCPyV (61). It is thought that individuals are infected early in childhood, and virus is detectable in the urine of about 30% of healthy individuals without causing disease (154). JCPyV has a strict host tropism dictated by cellular species-specific and tissue-specific factors required for viral replication (68). Because JCPyV can only infect humans, in vitro studies have only been able to demonstrate JCPyV infection in primary human fetal glial cells (153, 189), human brain microvascular endothelial (HBMVE) cells (34) (Lazaga and Nerurkar, unpublished), and human renal proximal tubule epithelial (RPTE) cells (Lazaga et al., unpublished data). These in vitro systems have been important in elucidating the basic molecular virology of JCPyV and testing for various antiviral compounds (251). JCPyV infection in nonhuman primates, owl monkeys (*Aotus* trivirgatus) and squirrel monkey *(Saimiri sciureus*), fail to demonstrate productive infection, as assessed by the lack of TAg and VP1 expression (251). The lack of a suitable animal model for JCPyV infection and PML has been a direct result of the inability of JCPyV to productively infect and replicate in non-human hosts. Therefore, data on potential primary site(s) of infection, areas of latency and reactivation, and the mechanisms involved in rearrangement during immunosuppression has been limited due to the absence of a JCPyV animal model.

Recently, a novel murine model, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) reconstituted with human hematopoietic stem cells, coined ‘humanized NSG mice’, has been employed to study human-specific pathogens (144, 214, 232, 257). In a quest to develop and characterize an animal infection model for JCPyV, we inoculated humanized NSG mice with JCPyV and evaluated the course of infection. The prospect of using NSG mice to study JCPyV infection is supported by the idea that the bone marrow (227) is a likely site of latency and that JCPyV can infect human B cells (31). Therefore, we hypothesize that humanized NSG mice can be productively infected by JCPyV.

Results

Characterization of humanized mice

Upon receiving the NSG mice, peripheral blood was collected from tail vein and blood leukocytes were tested for human CD45 by flow cytometry. The NSG mice used in this study were human CD45+, where the total percent of human leukocytes in the blood prior to inoculation ranged from 35-57%. Human immune cell lineage engraftment is expressed as the frequency of human CD45 cells, with CD19+ B cells ranging from 45-68%, CD4+ T cells ranging from 14-23%, and CD8+ T cells ranging from 8-14% (Fig. 1A). Mice were also analyzed for human immune cell engraftment 2 months after inoculation, where the total percent of human CD45+ cells ranged from 45-71% (Fig. 1B).

JCPyV infects humanized NSG mice

To test the presence of JCPyV DNA in humanized NSG mice, 30 week old female mice were inoculated via tail vein with either a single dose of 5000 HAU archetype or rearranged Mad-1 JCPyV and blood was first collected via tail vein 3 days after inoculation and once a week thereafter. JCPyV DNA was detected and quantitated from whole blood using JCPyV TAg and VP1 qPCR. All mice were positive for either JCPyV TAg and/or VP1 DNA in blood (Table 1.) up to two weeks after inoculation, with virus load peaking at 7 days after inoculation. JCPyV TAg DNA was detected in one of five mice at day 3 after inoculation. JCPyV TAg DNA copies detected on day 7 included one archetype JCPyV infected mouse with $4.33x10^5$ copies per µg of DNA and two Mad-1 JCPyV infected mice with $6.86x10^3$ and $8.99x10^5$ copies per µg of DNA. JCPyV VP1 was detected in all archetype JCPyV inoculated mice ranging from $1.32x10^2$ to $4.95x10^3$ copies per µg of DNA and one Mad-1 JCPyV inoculated mouse with 8 copies per µg of DNA detected on day 7. By day 14, JCPyV TAg DNA was detected in all archetype JCPyV

inoculated mice with DNA detected ranging from 2 to $1.53x10^5$ copies per µg of DNA, while Mad-1 JCPyV DNA was detected in one mouse with $2.19x10^2$ copies per µg of DNA. On day 14 two archetype JCPyV infected mice had detectable JCPyV VP1 DNA at 1 and 3 copies per µg of DNA. All blood samples were JCPyV RNA negative at all time points after inoculation.

To examine if humanized NSG mice shed virus after inoculation, urine was collected from mice on days 3, 5, 10, 14, 21, and at least once a week thereafter and up to 98 days after inoculation (Table 2.). The quantity of JCPyV DNA detected in the urine of archetype JCPyV infected mice ranged from $4.86x10^3$ to $6.32x10^7$ copies per µL of urine. The quantity of JCPyV DNA detected in the urine of Mad-1 infected mice ranged from 261 to $4.64x10^3$ copies per µL of urine. Although JCPyV detected in the blood and the urine did not coincide, all animals had detectable JC viral DNA present in blood or urine within the first 2 weeks of inoculation. JCPyV was not detectable in urine in either archetype or Mad-1 infected animals after 2 weeks of inoculation and JC viral DNA was not detected in the blood or urine of the control animal at any time during the experiment.

To further verify that JCPyV infects human CD45+ engrafted cells, JCPyV TAg protein was detected by flow cytometry. On day 7 after inoculation, JCPyV TAg protein was observed in human immune cells engrafted in three of five NSG mice, two archetype inoculated mice and one Mad-1 infected mouse (Fig 2.). As in human infection, mice were asymptomatic when inoculated with either type of JCPyV.

To determine if JCPyV was sequestered in tissues of humanized NSG mice, organs were analyzed for the presence of JCPyV viral DNA. Kidney, brain and bone marrow were collected

and analyzed using JCPyV TAg and VP1 qPCR. Of the three archetype inoculated humanized NSG mice, JCPyV viral DNA was detected in the kidney of two mice, whereas JCPyV viral DNA was detected in both Mad-1 inoculated humanized NSG mouse kidneys. JCPyV viral DNA was not detected in the control animal kidney. Brain and bone marrow were also negative for JCPyV viral DNA in all animals.

Discussion

Although it has been determined that JCPyV is the causative agent of PML, the route of primary infection and viral dissemination has yet to be fully elucidated. Although *in vitro* systems have been important in elucidating the basic molecular virology of JCPyV, understanding the natural history of JCPyV infection can only be answered utilizing a suitable animal model. To better understand the mechanisms that lead to JCPyV infection and dissemination, our goal was to determine JCPyV infection of human lymphocytes engrafted in a humanized NSG mouse model.

JCPyV is a ubiquitous virus thought to asymptomatically infect individuals early in life resulting in a subacute chronic infection of the kidney. In approximately 30% of individuals, virus is shed in the urine. Like for humans, JCPyV DNA was detected in the urine of mice for up to two weeks after infection and in the kidney after necropsy. It is thought that JCPyV disseminates throughout the body by a hematogenous route of infection, finding its way to secondary sites like the tonsils, brain, kidney, and bone marrow. Previously published data have demonstrated the infection of B cells by JCPyV in vitro (31) and the presence of JCPyV infected B cells in vivo (166). Our data demonstrates a proof of concept where human B cells in a humanized NSG mouse model can be infected with JCPyV. Detection of JCPyV DNA in the kidney after necropsy may demonstrate sequestration of either JCPyV associated B cells and/or cell free virus. Because JCPyV is a human-specific pathogen, the presence of JCPyV DNA in the kidney of inoculated animals is most likely due to virus being sequestered prior to being excreted in the urine. Furthermore, the absence of JCPyV DNA in the brain might suggest that trafficking of JCPyV infected B cells and/or cell free virus across the blood brain barrier may be restricted due to yet to be determined mechanisms. The absence of JCPyV DNA in other organs may be due the low levels of replication of JCPyV in B cells and the ability of JCPyV infected B cells to traffic

to secondary site and either sequester or find resident cells to infect due to the inability of JCPyV to infect murine cells. Although this model has limitations, such as a lack of persistent infection and sequestration in organs due to the apparent inability of JCPyV to infect murine cells, NSG mice could be used as a model to further study JCPyV immune responses and infection in the blood. A recent report demonstrated the generation of both humoral and cellular immune responses, although at low levels, against Mad-4 and CY JCPyV in NSG mice with engrafted human thymus (226). Furthermore, studies examining JCPyV in the blood have yielded inconsistent results. There are conflicting reports regarding the detection of JCPyV DNA by PCR in the PBMC in immunocompetent individuals, ranging from 0-83% (56, 233). In HIV-infected individuals without PML, JCPyV DNA detected in PBMCs varied from 0 -38%, while in patients with advanced AIDS and PML (52, 58, 59, 188, 233), JCPyV DNA was found in the PBMCs of 75-89% of these individuals (72, 233). Such reports, as well as our current data, lay the foundation for future studies where NSG mice could be used to study the immune response, viremia, and viruria in JCPyV and HIV coinfection, as well as in other cases of immunosuppression, such as during the use of monoclonal antibodies.

Materials and Methods

Humanized NSG mice

Humanized NOD/scid-IL-2Rγ$_c^{null}$ mice were obtained from The Jackson Laboratory and housed in a specific-pathogen-free animal facility at the John A. Burns School of Medicine, University of Hawaii at Manoa. All mice were maintained in sterile isolator cages, and fed sterile food and water.

Flow cytometry

Flow cytometry was conducted for the identification of CD45+ human peripheral blood leukocytes from 29-week old NSG mice before inoculation. Flow cytometry was conducted for the identification of JCPyV TAg in peripheral blood of NSG mice one week post JCPyV inoculation. Peripheral blood was collected from tail vein in EDTA-coated tubes. Blood leukocytes were tested for human pan-CD45, CD3, CD4, CD8, and CD19 markers as a five-color combination. Antibodies were obtained from Invitrogen (CD45, Cat# MHCD4501), BD Biosciences (CD8, Cat# 341051 and CD19, Cat# 555413), and CALTAG Laboratories (CD3, Cat# MHCD0305 and CD4, Cat# MHCD0417). Results were expressed as percentages of total number of gated lymphocytes.

Immunohistochemistry

Brains and kidneys were removed immediately after euthanasia. Brain and kidney from each mouse was fixed in 4% paraformaldehyde overnight, followed by an overnight incubation in 30% sucrose-PBS overnight. Brains and kidneys were then frozen in OCT compound.

JCPyV inoculation

Archetype JCPyV was isolated from a healthy volunteer and propagated in COS-7 cells (Lazaga and Nerurkar, unpublished), while Mad-1 JCPyV was propagated in PHFG cells. Virus titers were determined by qPCR and HA assay. At 30 weeks of age, female mice were injected via tail vein with either a single dose of 5,000 HAU archetype JCPyV or 5,000 HAU rearranged Mad-1 JCPyV. Blood was first collected via tail vein 3 days after inoculation and once a week thereafter for 12 weeks. Urine was collected on days 3, 5, 10, 14, and once a week thereafter.

DNA and RNA extraction and quantitative analysis of viral DNA

DNA was extracted from mock- and JCPyV-inoculated mice blood and urine using the QIAamp DNA Mini Kit (Cat #51306, Qiagen, CA). The DNeasy Blood and Tissue Kit (Cat #69504, Qiagen, CA) was used following the manufacturer's instructions for DNA extraction from organs. Total RNA from blood was extracted using the RNeasy Protect Animal Blood Kit (Cat #73224, Qiagen, CA) and cDNA was synthesized from 1 µg of toal RNA using iScript cDNA synthesis kit (Cat#170-8890, Bio-Rad) following the instructions provided by the manufacturer. JCPyV DNA or cDNA were amplified using 6 µL of template, 10 pmol each of forward and reverse primers, and probe specific for JCPyV TAg and for VP1 genes in a final reaction volume of 20 µL, as described previously (34). Thermal cycling conditions were followed as described previously (35). Real-time PCR was conducted using an Applied Biosystems 7500 Real-time PCR Detection system. Analysis was conducted via Applied Biosystems 7500 Software v2.0.5. All values above 1 copy was considered positive (226).

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Figure Legends

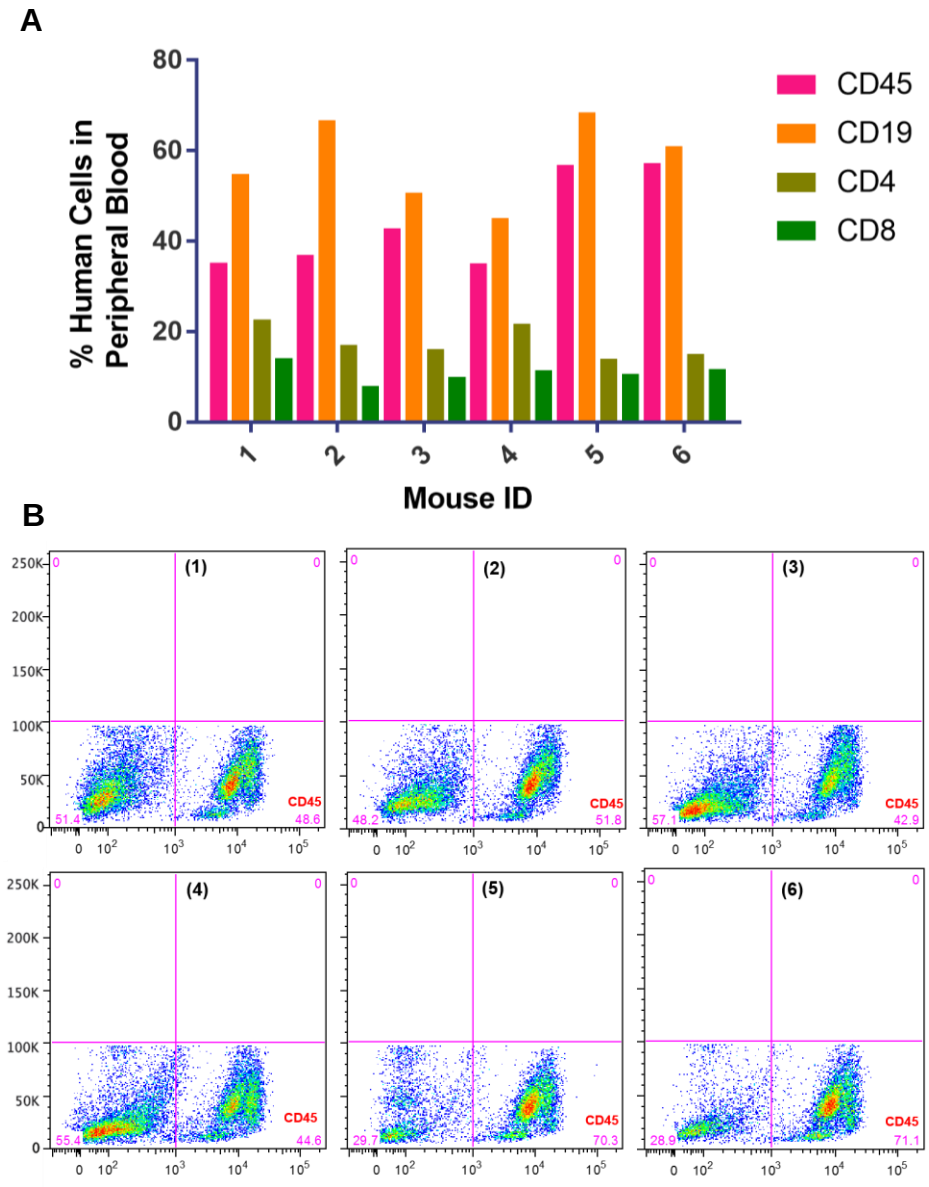

Figure 1. Characterization of humanized NSG mice. Mice were bled to detect human cell engraftment. (A) Cells stained with antibodies against B cell marker CD19 and T cell markers CD4 and CD8 expressed as frequency of human CD45 cells. (B) Peripheral blood cells were stained with antibodies against human panleukocyte marker CD45 from 29 week old NSG mice when first received. The percent of positive cells when gated for CD45 is indicated in the bottom right of each panel.

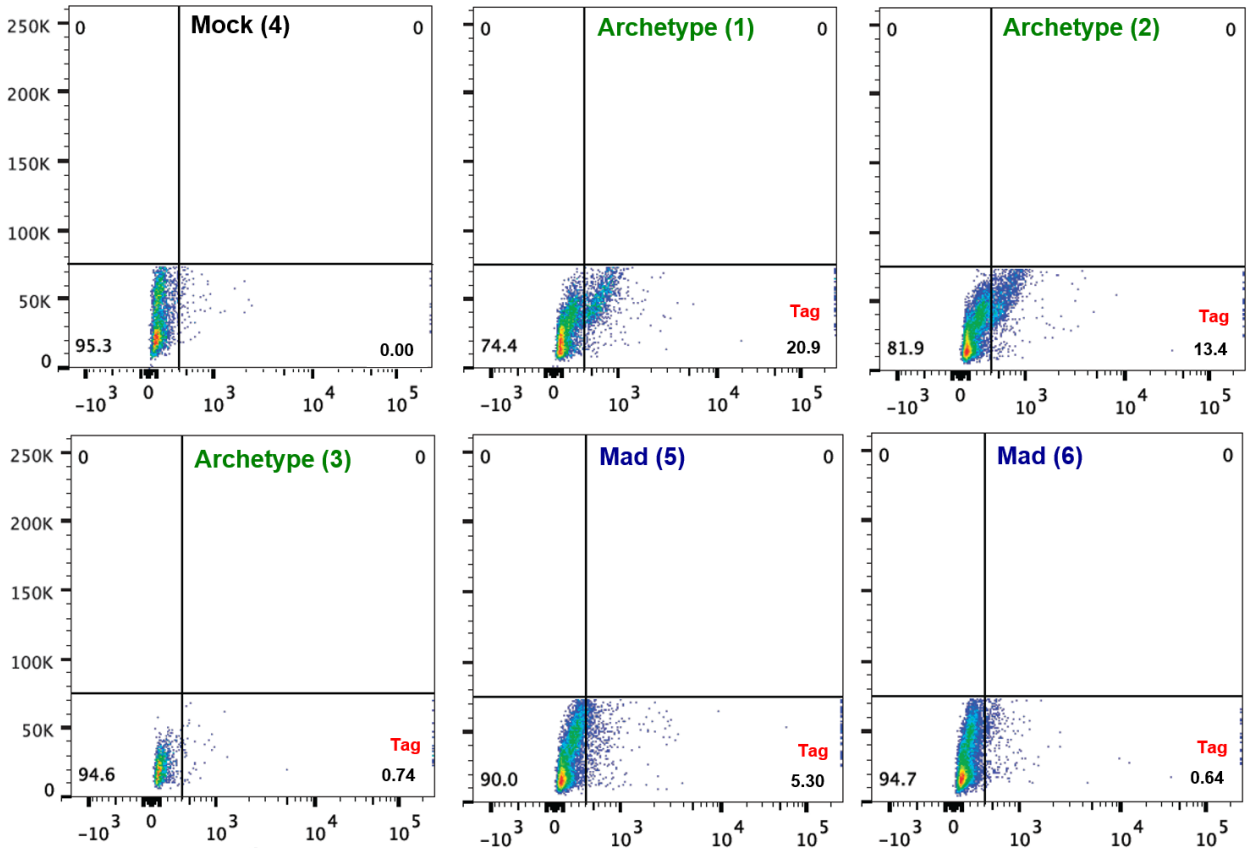

Figure 2. Detection of JCPyV TAg protein in peripheral blood of humanized NSG mice.

Peripheral blood was collected one week post-infection and cells were stained for intracellular JCPyV TAg. The percent of JCPyV TAg positive cells by intracellular staining is indicated in the bottom right of each panel.

Table 1. Detection of JCPyV TAg and VP1 DNA in peripheral blood of humanized NSG mice.

Mice ID		D3	D7	D14	D21	D28
4	TAg	ND	ND	ND	ND	ND
	VP1	ND	ND	ND	ND	ND
1	TAg	ND	10^5*	10^1	ND	ND
	VP1	ND	ND	10^0	ND	ND
2	TAg	ND	ND	10^3	ND	ND
	VP1	ND	10^3	10^1	ND	ND
3	TAg	ND	ND	10^3	ND	ND
	VP1	ND	10^3	ND	ND	ND
5	TAg	10^3	10^5	10^2	ND	ND
	VP1	ND	ND	ND	ND	ND
6	TAg	ND	10^3	ND	ND	ND
	VP1	ND	10^1	ND	ND	ND

*copies per µg of DNA; ND, not detected

Table 2. Detection of JCPyV TAg DNA in urine of humanized NSG mice.

Mice ID	D3	D5	D10	D14	D21
4	NC	NC	NC	ND	ND
1	NC	NC	10^{2}*	ND	ND
2	10^{2}	10^{3}	10^{7}	ND	ND
3	10^{2}	NC	10^{2}	ND	ND
5	NC	10^{3}	NC	ND	ND
6	10^{2}	10^{3}	10^{2}	ND	ND

*copies per µL of urine; ND, not detected; NC, not collected

Table 3. Detection of JCPyV TAg and VP1 DNA in organs of humanized NSG mice.

Mice ID		Kidney
4	TAg	ND
	VP1	ND
1	TAg	10^3
	VP1	ND
2	TAg	ND
	VP1	ND
3	TAg	10^2
	VP1	ND
5	TAg	10^3
	VP1	10^4
6	TAg	10^4
	VP1	10^3

*copies per µg of DNA; ND, not detected. Brain and bone marrow were negative for JCPyV TAg and VP1 DNA.

CHAPTER 6

OVERVIEW, LIMITATIONS, AND FUTURE DIRECTIONS

Overview and limitations

The ubiquitous human polyomavirus JC (JCPyV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (5, 195). Although rare in the general healthy population, in immunocompromised individuals, PML can result in severe disability or death caused by the lytic infection of oligodendrocytes (24, 196). If left unmanaged, the mortality rate is 30-50% within three months of diagnosis and although intervention can improve the chance of survival, it is likely that some significant neurological deficits will occur (24). PML is primarily seen in patients with HIV/AIDS, with approximately 5% of HIV/AIDS patients succumbing to PML, but has also been described in case reports in individuals with hematologic malignancies and individuals taking immunosuppressive or immunomodulatory therapies (148, 161, 216, 238). Of the best described instances of immunomodulatory therapies associated with PML is natalizumab (Tysabri®) used to treat multiple sclerosis patients. As of September 2016, approximately 161,300 patients received natalizumab, with 698 treated patients with a confirmed PML diagnosis (46). Interestingly, no PML cases have been reported in MS patients prior to the introduction of natalizumab.

JCPyV can be categorized into two types based on the structure of the non-coding control region (NCCR), the archetypal form, which contains 6 regions designated A-F, and the rearranged forms, which contains deletions and/or duplications (138, 260). Archetype JCPyV is largely detected in the kidney and shed in the urine in approximately 30% of immunocompetent individuals, while the rearranged form is predominantly detected in the brain and CSF of PML patients (61, 133, 154). Because asymptomatic shedding of JCPyV in the urine can be seen in both healthy individuals and immunosuppressed patients (4, 121, 122) the kidney is thought to be the major organ of JCPyV persistence during latency (39, 123). While the archetypal form of

the virus is found within the kidneys and urine of healthy individuals and individuals affected with PML (139, 260), the rearranged form is predominantly found within the cerebral spinal fluid (CSF) and brain tissues of patients with PML. Although the rearrangement of the JCPyV NCCR is important in the pathogenesis of PML, the mechanism by which this rearrangement occurs remains unknown (150). However, it is thought that rearrangement of the NCCR and point mutations in the capsid protein VP1 of archetype strains are a result of active replication (70, 113, 138, 187, 263).

Understanding the mechanisms underlying the development of PML has been hampered by the inability to conclusively delineate the sites of JCPyV latency and reactivation in humans, the inability to demonstrate rearrangement of archetype JCPyV in an *in vitro* replication model, and the absence of an *in vivo* animal model to study JCPyV pathogenesis. The long-term objective of the described research was to delineate the natural history of archetype JCPyV infection, reactivation, and rearrangement for evidence based treatment for PML.

Chapter 3 is dedicated to understanding the infection of archetype JCPyV in primary cells and to decipher how NCCR rearrangement may be induced *in vitro*. JCPyV has been detected in different tissues and organs in the human body including the tonsils (166, 168), kidney (227, 261), bone marrow (227), brain (227), spleen (227) and gastrointestinal tract (192), however, it is not clear which specific cell type(s) within the tissues and organs infected with JCPyV are permissive to infection and therefore a milieu to archetype JCPyV reactivation and rearrangement (49, 114, 247). To date, no experimental studies have been conducted to demonstrate infection of urine-derived archetype JCPyV in RPTE cells.

The difficulty in delineating the cell types susceptible to archetype JCPyV infection has been a result of its restricted host cell range *in vitro* (70, 94, 114, 181). To address issues with the limited host cell tropism that JCPyV displays, studies have either focused on, but not limited to, using transformed cell lines to drive the replication of JCPyV and/or introducing JCPyV DNA via a plasmid based system. A previous study demonstrated the expression of JCPyV TAg protein in RPTE cells but did not demonstrate the presence of DNA, RNA, or JCPyV virions (152). Although transfection can address the involvement of intracellular components, like DNA-binding proteins, in JCPyV transcription and DNA replication, transfection bypasses the question of binding potential of JCPyV to host cell receptors involved in entry of permissive cell types.

The host cell range of archetype JCPyV is strictly restricted in cultured cells, where researchers have demonstrated poor to moderate replication of archetype JCPyV in transformed cell lines, such as PHFG cells transformed with an origin-defective mutant of simian virus 40 (POJ-19) and simian kidney cells transformed with an origin-defective mutant of SV40 (COS-7) cells, respectively (50, 95). *In vitro* data indicates that various archetype JCPyV DNA clones can initiate efficient virus replication with the conservation of the NCCR after transfection in COS-7 cells (95). In contrast, it has been demonstrated that rearranged Mad-1 JCPyV can efficiently replicate in primary cells, including PHFG and HBMVE cells (35). Therefore, it has yet to be determined if archetype JCPyV can infect and replicate in primary RPTE cells. Our data, for the first time, clearly demonstrated the productive infection of archetype JCPyV in RPTE cells.

The exact mechanisms and events leading to NCCR rearrangement has yet to be determined. It has been suggested that the pathogenic rearranged form may be generated during virus replication, yielding the ability to acquire new tissue tropism and greater pathogenic potential

(49, 201). It has been established that functional TAg is required for JCPyV replication. This has been confirmed by the observation that mutations in the TAg-coding region of JCPyV cannot commence a lytic infection.

Utilization of COS-7 cells to produce large amounts of progeny JCPyV after transfection with conservation of the NCCR in DNA has been demonstrated (94). Archetype JCPyV transfected COS-7 (94), COS-7 cells constitutively expressing HIV-1 Tat (COS-tat) (179), and primary human fetal glial cells constitutively expressing JCPyV TAg (POJ-19) have been subjected to long term cultures (49), up to 72 days after transfection, to demonstrate continuous production of JCPyV progeny, but did not demonstrate rearrangement of the NCCR. We have recently demonstrated *in vitro* rearrangement of urine-derived archetype JCPyV after infection in COS-7 cells 645 days after infection, thus *in vitro* rearrangement of archetype JCPyV is possible in transformed cells expressing TAg.

TAg contains several intrinsic biochemical activities and binds specific cellular proteins required for JCPyV replication. The N terminus of TAg contains a DnaJ domain, which contributes to efficient viral replication, although it is not clear how this materializes (27). In addition to the DnaJ domain, TAg contains a retinoblastoma-associated protein (RB)-binding LXCXE motif, a threonine-proline-proline-lysine (TPPK) motif, a nuclear-localization sequence (NLS), a DNA-binding domain (DBD), and a helicase domain. The functions of these domains and motifs have been previously described to play important roles in the replication of JCPyV and /or other polyomaviruses. In short, the J domain cooperates with the LXCXE motif to disrupt the interaction between RB and the E2F family transcription factors in order to promote cell cycle entry and progression (224). The phosphorylation of the threonine residue in the TPPK motif

has been demonstrated to be required for TAg-mediated viral DNA replication (248), while the NLS binds specifically to KPNA family importin homologues (51). The DBD and helicase domains are required for viral replication and recruit cellular DNA replication factors. The DBD binds the replication factors DNA polymerase-α catalytic subunit (POLA), the replication protein A complex (RPA), and the DNA primase complex (PRIM). Lastly, the helicase domain binds the molecules EP300, CREBBP, p53, and DNA topoisomerase 1 (TOP1) (51).

Due to the importance of TAg in JCPyV replication, it is not surprising that constant expression of TAg would eventually drive changes in the JCPyV genome over time. In a recent study looking at viral mutation rates, it was estimated that the mutation rates of DNA viruses presented as substitutions per nucleotide per cell infection (s/n/c) ranged from 10^{-8} to 10^{-6} s/n/c (207). Thus, the chances of mutations occurring in the NCCR of archetype JCPyV would increase over time due to the constant driven replication in COS-7 cells, but does not explain the mechanism in which *in vitro* rearrangement occurs. This system has its limitations including the length of time to prove rearrangement and utilizing a transformed cell line. Thus, future studies will be focused on using agents to induce NCCR rearrangements, such as dimethyl fumurate, fingolimod, and leflunomide, all of which have been associated with PML. Furthermore, we will identify the point in which this rearrangement occurred by analyzing previously frozen lysates by deep sequencing and identifying possible transcription factors that contributed to this rearrangement.

Chapter 4 is dedicated to understanding the effect of PML associated JCPyV VP1 mutations on JCPyV replication kinetics in HBMVE cells. A discrepancy between the high prevalence of JCPyV and the low incidence of PML in the human population suggests the progression from

asymptomatic infection to PML could be controlled by a unique viral characteristic. Recent work has demonstrated amino acid changes to the viral capsid protein VP1 resulted in accelerated evolution in viral sequences isolated form PML patients but not in sequences isolated from healthy individuals (225). In addition, VP1 derived virus-like particles (VLP) exhibiting mutations resulted in diminished hemagglutination ability, demonstrated different ganglioside specificity, and abolished binding to different peripheral cell types compared with wild-type VLPs (87). Unfortunately, the limitation to this study was the inability to demonstrate replication of JCPyV VP1 mutants in HBMVE cells, and therefore we could not study the loss of hemagglutination activity because virus was not produced after transfection. Therefore, it still remains unclear whether alterations to JCPyV VP1 will exhibit changes in viral DNA replication activity, infectious virus production, and NCCR rearrangement.

The artificial insertion of exogenous viral DNA into cells via transfection is an important, well-established tool, however, our results highlights the importance in understanding the natural process of infection, whereby initial binding of virus might trigger downstream molecules important to the JCPyV lifecycle. Utilizing similar methods to propagate urine-derived archetype JCPyV, we were able to propagate infectious CY virions to corroborate that CY, like urine-derived archetype JCPyV, can infect HBMVE cells. Future studies will address this by propagating infectious JCPyV VP1 mutant virions by transfecting COS-7 cells, instead of HBMVE cells, as seen with our ability to propagate infectious CY virions after transfecting COS-7 cells. It would be of interest to utilize our established method of propagation to create infectious virions for JCPyV VP1 mutants.

Lastly, chapter 5 is dedicated to developing an *in vivo* JCPyV infection animal model. Recently, a novel murine model, NOD scid gamma (NSG) reconstituted with human hematopoietic stem cells, coined 'humanized NSG mice', has been employed to study human-specific pathogens (162). 12 weeks after engraftment with human CD34+ hematopoietic stem cells, NSG mice display engraftment of mature human white blood cells, human CD45+, including human B cells, human CD19+. JCPyV, a human specific pathogen, has been shown to infect human EBV transformed B cells (31). Although straightforward, we demonstrated archetype and rearranged JCPyV infection in human B cells, albeit at low levels, and excretion of JCPyV DNA in the urine of NSG mice. While this model has its limitations, including the inability to demonstrate pathogenesis and persistent infection in organs due to the inability of JCPyV to infect murine cells, NSG mice could be used as a model to further study JCPyV immune responses and infection in the blood.

Future plans

The scope of this thesis, simply put, was to understand archetype JCPyV infection. This objective was satisfied by demonstrating the infection of archetype JCPyV in *in vitro* and *in vivo* models, delineating the cellular tropism of archetype JCPyV, and trying to understand possible mechanisms of reactivation and rearrangement leading to the pathogenic rearranged form of JCPyV. To strengthen our findings, future studies are focused on determining the cellular factors, including but not limited to, transcription factors and cytidine deaminases, that can contribute to JCPyV replication and rearrangement.

To better understand how transcription factors play a role in archetype JCPyV reactivation and rearrangement, we will compare and contrast transcription factors that can bind to the NCCR of

archetype, Mad-1 rearranged, and D645 rearranged strains of JCPyV by predictive modeling. We will determine the expression of transcription factors in JCPyV permissive and non-permissive cells. Once we identify the transcription factors that contribute to virus reactivation, we will employ site-directed mutagenesis of archetype JCPyV NCCR to abrogate the binding of transcription factors and siRNA knockdown of transcription factors to demonstrate loss of function and disruption of JCPyV replication.

It has been established that cell type specificity of JCPyV within human cells occurs at the transcriptional level. The regulation of transcription is dependent on the sequence of the NCCR, as well as the availability of host transcription factors, which are the determining factor in both the start sites of early transcription, as well as the quantity of TAg produced (70). Unlike other human DNA-containing viruses, such as herpesviruses, JCPyV does not bring transcriptional activating proteins into newly infected cells. Therefore, although host cell factors are the determining factor in directing early transcription, the exact profile of transcription factors involved in reactivation and rearrangement remains elusive.

Until now, there has been a crucial need for *in vitro* systems mimicking JCPyV infection to address key questions in JCPyV biology. By understating what cells are conducive to JCPyV infection, replication, and reactivation, we can target parts of the JCPyV life cycle for therapeutics to prevent or treat PML, including JCPyV TAg and/or host transcription factors that are needed to initiate transcription. This dissertation has provided new molecular information for understanding JCPyV tropism, archetype JCPyV propagation, and more importantly identified key cells conducive to archetype JCPyV infection, which has never been described. Therefore, the findings in this dissertation can further advance the field of JCPyV by providing novel

insights in establishing physiologically authentic infection models and co-culturing models, (i.e. blood-brain barrier model) to test potential therapeutics.

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